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PhD thesis summary

Molekulární patologie vybraných dědičných hyperbilirubinémií

Molecular pathology of selected inherited hyperbilirubinemias

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The full text of the thesis will be available at least five days before the PhD. defense at the Department of Science and Research and International Relations of the 1st Faculty of Medicine, Charles University in Prague.

CONTENT

ABSTRAKT

Dědičné hyperbilirubinémie představují skupinu metabolických onemocnění, charakterizovaných zvýšenou hladinu celkového bilirubinu nebo jeho konjugované frakce v séru. Většina těchto hyperbilirubinémií je děděná autosomálně recesivně a jsou manifestovány zejména v mladém věku. Zvýšený bilirubin je zapříčiněn poruchami v některém z enzymů metabolické dráhy hemové degradace, ať už jeho konjugace (*UGT1A1*) nebo transportu (*ABCC2, OATP1B1, OATP1B3*). Všechny proteinové produkty těchto genů se účastní nejen eliminace bilirubinu, ale take různých dalších substrátů. Studie těchto protein mají tedy široký farmakogenomický vliv. Tato studie popisuje molekulární podstatu dědičných hyperbilirubinémií u kavkazské a romské populace, srovnává klinická a biochemická data s výsledky molekulární genetiky. Dále popisujeme vliv současného výskytu variant c.-3279T>G a g.175492_175493insTA na hladinu celkového sérového bilirubinu. Stanovili jsme vazebnou nerovnováhu obou variant lokalizovaných v promotorové oblasti genu UGT1A1. Zároveň jsme ověřili konzistenci populačních dat s výsledky v literature.

Ve druhé studie jsme popsali vzácnou konjugovanou hyperbilirubinémii Dubin-Johnsonova typu u 7 romských rodin. Našli jsme novou variantu v *ABCC2* genu NG_011798.1:c.[1013_1014delTG] a take dvojitý genetický defect – kombinaci Dubin-Johnsonova a Gilbertova szndromu. Popsali jsme novou mutaci v genu ABCC2 a našli společný haplotyp o velikosti 86 bp v blízkosti genu. Dále jsme charakterizovali exkreci izomerů koproporpfyrinu a srovnali data pacientů s DJS a dvojitou hereditární žloutenkou.

Klíčová slova:

bilirubin, žloutenka, hyperbilirubinemie, *UGT1A1, ABCC2*, izomery koproporfyrinu,

ABSTRACT

Inherited hyperbilirubinemias are a group of metabolic disorders, characterized by increased levels of total serum bilirubin or its conjugated fraction. Most of these hyperbilirubinemias are inherited autosomal recessively and are manifested in young age. Increased bilirubin reflects the genetic disturbances in one of the enzymes of heme degradation pathway, the defect of bilirubin conjugation (*UGT1A1 gene*) or its transport (*ABCC2, OATP1B1, OATP1B3*). All of these proteins are involved not only in elimination of bilirubin, but various substrates; therefore the performed studies have a great pharmacogenomics impact. We have studied the molecular pathology of hereditary hyperbilirubinemias in Caucasian and Roma population and to compare the clinical and biochemical results with the molecular genetic data. We described the impact of compound defect of c.-3279T>G and g.175492_175493insTA on total serum bilirubin and calculated the linkage disequlibrium of these two variants in promoter region of *UGT1A1* gene. We also verified, that the population distribution of both variants is in concordance with the literature.

In our second study, we have described the rare conjugated hyperbilirubinemia Dubin-Johnson type among 7 Roma families. We have found a novel variant NG_011798.1:c.[1013_1014delTG] together with the dual genetic defect – a combination of Dubin-Johnson and Gilbert`s syndrome. We have described a founder effect of the mutation in *ABCC2* and found a common haplotype of 86 bp encompassing the gene. We have also characterized the excretion of coproporphyrin isomers in patients with DJS and compared it with those with dual hereditary jaundice.

KEYWORDS:

Bilirubin, jaundice, hyperbilirubinemia, *UGT1A1, ABCC2*, coproporphyrin isomers

INTRODUCTION

Bilirubin

Bilirubin is the product of heme catabolism. In humans, a major source of bilirubin is heme derived from hemoglobin released from senescent erythrocytes (~ 80%) (London et al, 1950). Other sources of heme are hemoproteins such as peroxidases, catalases; or myoglobin. Erythrocytes lifespan is about 120 days and a daily production of bilirubin is 250 – 400 mg. Even though every eukaryotic cell is able to produce bilirubin, the majority of heme degradation occurs in the reticuloendothelial system of spleen and liver.

Heme degradation to bilirubin occurs in several steps:

1. Heme oxidation by heme oxygenase 1
2. Reduction of biliverdin to bilirubin
3. Conjugation of bilirubin with glucuronic acid
4. Bilirubin transport
5. Bilirubin catabolism

Chemical name of bilirubin is 1,8-dioxo-1,3,6,7-tetramethyl-2,8-divinylbiladiene-a,c-dipropionic acid. Bilirubin is a water insoluble yelowish, weekly acidic compound, having antioxidant properties on one side and neurotoxic properties on another. Water insolubility of bilirubin molecule is a key issue in bilirubin elimination in vivo. As none of the organisms want to store its toxic products, the solution is in making bilirubin soluble by its conjugation with glucuronic acid.

Inherited hyperbilirubinemias are a group of metabolic disorders, characterized by elevated levels of total and/or conjugated bilirubin in blood. They result from the genetic disturbances in some of the genes enconding proteins of bilirubin degradation pathway. Most of inherited hyperbilirubinemias have autosomal recessive inheritence (Bosma et al, 1995)

Jaundice and its therapy

Jaundice appears as a clinical sign of elevated bilirubin in adults when total bilirubin > 50 and mostly around 80 umol/L, therefore it is a clinical sign of several inherited hyperbilirubinemias (Silbertnagl et al, 2009). It is presented as a yellowish pigmentation of skin and/or sclerae. The most severe jaundice occurs in patients with Crigler-Najjar syndrome type I, which, without the early treatment, can be fatal. On the other side, in patients with Crigler-Najjar syndrome type II, the hyperbilirubinemia and jaundice are milder. Occasionally, jaundice is presented in Dubin-Johnson, Rotor or Gilbert` s syndrome.

The most often used therapy of jaundice is a phototherapy The principle of a phototherapy is in use of blue light is a change of bilirubin conformation from Z (trans) to E (cis) with resultig bilirubin isomers in ZE, EE or EZ conformation. These isomers lack internal hydrogen bonds, therefore making bilirubin more polar and unnecessary to be conjugated with glucuronic acid. (Mc Donagh et al 1972, Itoh et al 1985; Onishi et al 1980, Yokoyama et al 1984).

UGT1A1

UGT1A1 gene (uridine diphosphate glucuronosyltransferase) is a member of a broad enzymatic family of uridine diphosphate glucuronosyltransferases, strongly involved in metabolism of xenobiotics (Bosma, 1995). These enzymes protect organism from its toxic compounds by glucuronidation – the addition of the glycosyl residue to the small molecule. Resulting product such as bilirubin is water soluble and able to be eliminated by kidneys or by intestine. UGT1A1 mediates the conjugation of numerous substrates with glucuronic acid.

The defects in promoter regions of *UGT1A1* gene result in inherited hyperbilirubinemia, Gilbert´s syndrome (GS) (OMIM ID:143 500). GS is a hereditary, chronic, mild, benign, unconjugated hyperbilirubinemia resulting from impaired bilirubin clearance with otherwise normal liver function. The degree of hyperbilirubinemia is relatively mild, mostly less than 80 µmol/L (4.7 mg/dL) in adults (Bosma et al, 1995). In Caucasians, the frequency of GS is 5–10% (Owens and Evans, 1975). The most common variant is the TA insertion in the TATAA box of the UGT1A1 called UGT1A*28 or NG_002601.2:g.[175492_175493insTA].

Rare defects in a structural part of *UGT1A1* gene are associated with Crigler-Najjar syndrome I or II. The most severe inherited hyperbilirubinemia is Crigler-Najjar syndrome I (CNJ I) (OMIM 218 800) with the total serum bilirubin around 340-685 µmol/L or higher. The defect is caused by impaired UGT1A1, whose activity remains < 10%; Patients with CNJ I do not respond to phenobarbital treatment. Unlike CNJ I, in CNJ II the activity of mutated UGT1A1 is > 10 %, which enables its inducibility. The hyperbilirubinemia is from 60-340 µmol/L (Crigler and Najjar, 1952).

***ABCC2* gene**

Pathogenic genetic defects in ABCC2 gene result in conjugated hyperbilirubinemia Dubin-Johnson syndrome. Dubin-Johnson syndrome (DJS; OMIM #237500) is a rare autosomal recessive hyperbilirubinemia characterized by the absence of functional MRP2 protein at the canalicular membrane of hepatocytes (Mor Cohen 2001). To date, more than 40 variants in ABCC2 causing DJS have been described and several SNPs with pharmacogenomics importance (Keitel et al, 2003, Chen et al 1999, van Zanden et al 2005).

The clinical profile of DJS comprises of: (1) Conjugated hyperbilirubinemia in blood (> 7 µmol/L), (2) inverted ratio of excreted coproporphyrin isomers I to III in urine (> 80 % of isomer I), (3) prolonged visualization of liver by technetium (Tc-99)-labeled hepatobiliary iminodiacetic acid 99mTc-HIDA and, (4) abnormal deposits of melanin-like pigment in lysosomes of hepatocytes. Liver function remains otherwise normal.

OATP1B1 and OATP1B3

The other conjugated hyperbilirubinemia is Rotor syndrome, caused by a defects in two genes encoding transport proteins. The association of OATP1B1 and OATP1B3 with Rotor syndrome was found by Steeg et al in 2012 (Steeg et al, 2012). Inheritance is also autosomal recessive and the hallmark is inverted ratio of excreted coproporphyrins with a different profile than DJS.

AIMS OF THE STUDY

The aims of the study were to characterize the molecular pathology of hereditary hyperbilirubinemias in Caucasian and Roma population and to compare the clinical and biochemical results with the molecular genetic data.

1. To find the coincidence of c.-3279T>G variant in PBREM of *UGT1A1* and UGT1A*28 allele with TA insertion in TATAA box and its impact on serum bilirubin levels in probands with Gilbert`s syndrome and in healthy controls
2. To characterize the Dubin-Johnson syndrome on both molecular genetic and biochemical levels; To perform a mutation analysis of *ABCC2* in patients with DJS and its effect on bilirubin elimination
3. To study the excretion of coproporphyrin isomers in patients with DJS and its comparison with the genetic defect in *ABCC2*
4. To describe genetic and biochemical characteristics of Dual hereditary jaundice

MATERIAL AND METHODS

All studies were approved by Ethic Medical Committee, First Faculty of Medicine and General Faculty Hospital, Charles University in Prague. Informed consents were sign by all attendants of the studies.

DNA analyses Genomic DNA was extracted from 7 mL whole blood in EDTA using standard procedures. *ABCC2* gene was amplified using PCR, PP Master Mix polymerase with buffer (Top-Bio, Prague, Czech Republic) and 2.5 pM of primer; purified (Promega A9282 kit); and sequenced by ABI PRISM 3100 AVANT (Applied Biosystems, Big Dye Terminator 3.1 Sequencing Kit). Primer sequences according to Slachtova et al, 2015. Fragment analysis of TA insertion of TATA box of *UGT1A1* gene was performed (rs8175347, chr2:hg19:g.175492_175493insTA. PBREM enhancer variant c.-3279T>G (rs4124874; chr2:hg19:g.172270T>G) was amplified and assessed with RFLP as described previously (Maruo et al, 2004). All sequence variants were annotated according to reference sequences (NG_011798.1 for *ABCC2*, NG_002601.2 for *UGT1A1*), and new findings were submitted to ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar).

Coproporphyrin isomers analysis. Urine samples from 8 patients with jaundice were collected (100 mL per patient), and stored in dark at -80°C. Porphyrins and coproporphyrin isomers I and III were analyzed by HPLC with a fluorescence detector (excitation 405 nm, emission 620 nm), Chromsystems column (# 44100) and kit Porphyrins in Urine (# 44000, Chromsystems).

Microarray, haplotyping and variant´s age estimation. To assess a common haplotype, genomic DNA from 40 individuals was hybridized to Affymetrix Genome Wide Human SNP Array 6.0 according to manufacturer´s recommendation and further analysed. Genotype calling and loss of heterozygosity in 10q24 region was determined with Affymetrix Genotyping Console version 4.1. The genotyping data were entered in PLINK 1.07 and SNPs present on chromosome 10 were selected for further analyses. After performing standard quality checks, unphased genotype data of the whole cohort were entered into the Genotype Visualization and Algorithmic Tool (GEVALT) software, version 2. Phasing, linkage disequilibrium (LD) analysis and estimation of the haplotype block structure was done utilizing the Genotype Resolution and Block Identification using Likehood (GERBILT) algorithm. A haplotype block encompassing the site of c.1013_1014delTG variant in *ABCC2* gene included 21 SNPs. The age of the ancestral variant c.1013_1014delTG in Slovakian Roma population was estimated using a standard Luria - Delbrück algorithm modified by Austerlitz in Mathematica 8 using the Mathematica notebook kindly provided by F. Austerlitz. Variant`s age was estimated for a current population size of 400 000 – 450 000 Roma.

RESULTS

AIM 1 – Publication 1: **Slachtova L**, Kemlink D, Martasek P, Kabicek P. Does Bilirubin Level Correspond to Interaction of c.-3279T>G and A(TA)7TAA Variants in UGT1A1 Gene? Cell Mol Biol 2009, 55: 95-98

AIM 2, 3 and 4 – Publication 2: **Slachtova L**, Seda O, Behunova J, Mistrik M, Martasek P. *Genetic and biochemical study of Dual hereditary jaundice: Dubin-Johnson & Gilbert syndrome. Haplotyping and founder effect of deletion in ABCC2* Eur J Hum Gen 2015,

Biochemical blood tests included total and conjugated serum bilirubin, ALT and AST. ALT and AST ranged within normal levels, indicating the absence of liver damage. TBi in DJS patients was 18.8 - 72.2 µmol/L with cBi 9.5 - 55.2 µmol/L (Table 1). The percentage of cBi out of TBi in patients with dual defect DJS + GS were 28 %, 38 %, 43 % and 89 % (see Table 2). The last value 89 % was found in proband F1.IV/17 on medication with following TBi/cBi: 38/33; 70/55; and 35/31 µmol/L. Values of TBi and cBi of patients with the same *UGT1A1* and *ABCC2* haplotype varied (Table 1).

Molecular genetic analysis of the proband revealed the deletion in *ABCC2* gene c.1013_1014delTG responsible for Dubin-Johnson syndrome. The causal variant is located in the eighth exon and, *via* a frameshift, it leads to a premature stop codon p.(Val338Glufs14*)

resulting in a shortened protein product. Investigation of TATA box of *UGT1A1* of the same proband showed also NG_002601.2:g.[175492_175493insTA] causing Gilbert´s syndrome. Next, family members and probands were investigated for the defect in *ABCC2* gene with the total result of 17 homozygous patients with the deletion c.1013_1014delTG and 30 heterozygous carriers as evident from Figure 1. The presence of the same genetic defect in all seven seemingly unrelated families suggested a founder effect of c.1013_1014delTG among the investigated Roma families. Moreover, four patients with homozygous variant in *ABCC2* gene were also homozygous for the TA insertion in TATA box of *UGT1A1* gene with a genotype NG_011798.1:c.[1013_1014delTG]; NG_002601.2:g.[175492_175493insTA] (Table 1). All patients with homozygous TA insertion were also homozygotes for c.-3279T>G variant in PBREM.

Coproporphyrin isomers excretion. We analyzed coproporphyrin isomers in urine samples of 11 individuals: in eight patients with DJS caused by c.1013_1014delTG in *ABCC2* gene and in three heterozygous carriers. Significantly increased proportion of excreted coproporphyrin isomer I was observed in all DJS patients with values ranging from 89-100 %. In heterozygous carriers, the ratio of excreted isomer I was 43-57 %, compared to about 25-30 % in healthy controls (control data from literature). No differences were found in excrection of coproporphyrin isomers in patients with DJS compared to thouse with dual defect of DJS + GS.

Microarrays and haplotyping. The analysis of chromosome 10 genotypes in *ABCC2* region revealed a shared 86 kbp haplotype upstream and within *ABCC2* gene, transmitted together with the deletion c.1013_1014delTG. Furthermore, the loss of heterozygosity was found in all homozygous patients with DJS with use of CNV analysis from Affymetrix Genotyping Console. Loss of heterozygosity in DJS patients was in all cases in concordance with both genetic and biochemical data.

Estimation of variant´s age. Age of ancestral variant c.1013_1014delTG was estimated using the Luria-Delbrück algorithm modified by Austerlitz. Population size of Slovakian Roma inhabitants was set at 400 000 – 450 000 (Sproch, 2013) and the frequency of disease allele was estimated between 0.01 – 0.001. Using these data, our results show that the variant originated 7.1-7.4 generations ago, which corresponds to 177.5 – 185 years ago.

DISCUSSION

VI.I. Aim 1, Paper 1 - Does Bilirubin Level Correspond to Interaction of c.-3279T>G and A(TA)7TAA Variants in UGT1A1 Gene?

Functional studies show, that promoter variant c.-3279T>G, as well as A(TA)7TAA decrease the transcription of *UGT1A1* up to 60 %, resulting in consequently reduced glucuronidation of bilirubin and affecting its elimination as a toxic product (Bosma et al, 1995, Sugatani et al, 2002). Therefore, coincidence of both variants should lead to significant increase of hyperbilirubinemia. Combined genetic defect affecting glucuronidation of bilirubin and its elimination was described on the compound defects of A(TA)7TAA and other variants, located in a coding region of *UGT1A1* (Kamisako, 2004).

In our cohort of 101 patients and 84 controls from general Caucasian population we have studied the impact of coincidental occurrence of both genetic defect located in promoter region of *UGT1A1* on serum bilirubin and calculated the linkage disequlibrium of both variants. It is important to highlight, that presented data are describing genetic defects in Caucasian population. In Japanese population, the prevalent variant causing Gilbert`s syndrome is G211A (Koiwai et al, 1995).

The frequency of [(TA)7] allele in healthy controls was 32%, which responds to the data from literature (Bosma et al, 1995, Jirsa et al, 2006). Regarding c.-3279T>G variant, frequency of this variant was 39% among healthy population. As expected, significantly higher incidence of both variants was found in patients with GS – 99 % carried variant c.-3279T>G variant and 97 % had [(TA)7] allele. The linkage between both variants in controls was D´= 0.79, r^2 = 0.47 compared to linkage in patients with GS D´ = 1, but $r^2$was very low (0.32). The ODDs ratio of c.-3279T>G variant and Gilbert`s syndrome was OR 34.42, confirming the strong association of PBREM promoter variant and GS.

The linkage of c.-3279T>G and A(TA)7TAA in promoter region of UGT1A1 was previously suggested by Maruo et al (Maruo et al, 2004), who postulated the hypothesis, that the presence of both variants is necessary for the manifestation of Gilbert`s syndrome. However,

next study conducted by Czech authors Jirsa et al on a larger cohort showed, that even when the linkage of both variant is strong, it is not necessary for clinical manifestation of GS (Jirsa et al, 2006).

The effect of both promoter variants c.-3279T>G and A(TA)7TAA in *UGT1A1* on serum bilirubin is summarized in Table 2 (Slachtova et al, 2009).

	T/T	T/G	G/G
6/6	8,37 (±2,31)	8,47 (±0,81)	13
6/7	8,48 (±3,66)	10,58 (±4,39)	13,62 (±3,15)
7/7	0	12,3 (±4,53)	0

With the development of molecular genetic techniques, current data confirm the importance of various genetic defects on glucuronidation or bilirubin elimination. Several studies based on haplotyping techniques show the importance of genotyping variants involved in glucuronidation and elimination of toxic compounds. Bilirubin is transformed into its soluble form solely by UGT1A1. However, importance of haplotyping doesn`t involve the elimination of only bilirubin, but especially the clearance of other toxic compounds as the UGTs are the general drug metabolizing enzymes (Lankisch 2009).

VI.II. Aim 2 – Paper 2 - To characterize the Dubin-Johnson syndrome on both molecular genetic and biochemical levels; To perform a mutation analysis of *ABCC2* in patients with DJS and its effect on bilirubin elimination.

In literature, DJS has been characterized in a cohort of 101 Israeli patients, collecting biochemical, clinical and family history data (Shani et al, 1970). However, there is no study describing the defects on molecular genetic data, the relationship of mutations in *ABCC2,* impact of the mutated protein on direct excretion of bilirubin or coproporphyrin isomers.

Our study describes the biochemical and molecular genetics relationship on 56 investigated members from 7 Slovakian Roma families, which is the largest cohort described in Europe up

today. Our data suggest the common ancestral mutation allele with the origin of 178-185 years ago. In the beginning of our study, we hoped to obtain more variable cohort with various mutations, analyse the impact of the protein damage on cellular level and compared the data within the cohort. However, when analysing our data, we found, that the high incidence of rare Dubin-Johnson syndrome is caused by the effect of one founder mutation, and that all probands share the common haplotype of 86 kbp encompassing *ABCC2* gene. According our results and in concordance with literature, conjugated bilirubin is presented in all DJS patients with homozygous presence of c.1013_1014delTG had elevated cBi (9-55 µmol/L) as well as TBi (19-72 µmol/L). (Slachtova et al, 2015, Dubin and Johnson, 1952); however it is clear that the bilirubin levels are fluctuating even within the same haplotype, suggesting that bilirubin elimination does not reflect only the genotype, but also the influence of environmental factor, age of the probands or nutrition.

Table 1 A shared haplotype within the probands with DJS.

	I/1		**I/2**		**II/1**		**II/5**	
	father		**mother**		**daughter**		**son**	
rs2756115	T	C	T	C	C	C	C	C
rs17112219	G	G	G	G	G	G	G	G
rs3184991	T	T	T	T	T	T	T	T
rs2093354	A	A	A	A	A	A	A	A
rs2804412	G	G	G	G	G	G	G	G
rs2804409	T	T	T	T	T	T	T	T
rs2756095	A	A	A	A	A	A	A	A
rs7176201	C	C	C	C	C	C	C	C
rs4919395	G	A	G	A	A	A	A	A
rs2756103	A	C	A	C	C	C	C	C
rs4148389	A	G	A	G	G	G	G	G
rs17222744	A	A	A	A	A	A	A	A
rs2804398	A	T	A	T	T	T	T	T
rs2756109	T	T	T	T	T	T	T	T
rs11816875	G	G	G	G	G	G	G	G
rs2804397	G	G	G	G	G	G	G	G
c.1013_1014delTG	W	*	W	*	*	*	*	*
rs2273697	A	G	A	G	G	G	G	G
rs2002042	C	C	C	C	C	C	C	C
rs4148396	C	T	C	T	T	T	T	T

rs4148399	T	G	T	G	G	G	G	G
rs7476245	G	G	G	G	G	G	G	G

VI. III – Aim 3 and 4 – Paper 2 To study the excretion of coproporphyrin isomers in patients with DJS and to compare it with the genetic defect in *ABCC2;* to describe genetic and biochemical characteristics of Dual hereditary jaundice

Coproporphyrin isomers in urine are excreted as isomers I – IV with the most significant isomers I and III. In healthy adults, the proportion of coproporphyrin isomer III in urine is ~ 70-80 %, compared to ~ 20-30 % in DJS patients, who excrete about 80-97 % of coproporphyrin I. Under normal conditions, excretion of coproporphyrin isomers depends on maturation of hepatobiliary system and changes within the first days of newborn's life (Koskelo et al, Kondo et al 1976).

In our study, we have investigated coproporphyrin isomers in 8 patients with DJS caused by c.1013_1014delTG in *ABCC2* and all of them showed shifted ratio excreted coproporphyrin isomers in urine (89-100 %, Table 2). Several studies show up to > 90 % predominance of coproporphyrin I in urine of patients with DJS, but no data shows results of 100 % (Ben Ezzer et al, 1973, Shani et al, 1970). Our patient with 100% excretion of isomer I was one year old boy with total serum bilirubin 50 µmol/L and its conjugated fraction 21 µmol/L. Coproporphyrin isomers were analysed from two independent samples with the same result. Unafected heterozygotes secrete about 40% of isomer I compared to 20-30 % of isomer I in healthy controls. Average percentage of isomer I in our DJS carriers was 43-57 %, which is in concordance with a data from literature. Surprisingly, excretion pattern of coproporphyrin isomers in four patients with dual hereditary jaundice was the same as in other DJS patients with no defect in *UGT1A1* Patients with dual defect of NG_011798.1:c.[1013_1014delTG]; NM_000463.2:c.[-54_-53insTA] had the ratio of coproporphyrin I 92, 97, 99 and 100 % , compared to 89, 93, 96 and 98 % in DJS patients with only heterozygous NG_002601.2:g.175492_175493insTA and 89 % in DJS patient with the wild type *UGT1A* gene. This data suggest, that the excretion of coproporphyrin isomers depends not only on genotype of the patients but also on the other factors.

Dual hereditary jaundice has been described in a single case by other Czech authors. Our study aimed to collect more data on this rare condition, affecting both conjugation and transport of the bilirubin. In our study we are describing four DJS + GS patients. Supposed effect on the ratio of conjugated and excreted bilirubin is decreased amount of cBi available for its transport; therefore the percentage of cBi from TBi is < 50 % in DJS + GS compared to > 50 % in DJS only. We found a ratio of conjugated bilirubin fraction of 28 %, 38 %, 43 % and 89 %. The last value of 89 % is from a probands whose data were collected under medication, probably affecting the bilirubin clearance (Slachtova et al, 2015).

CONCLUSIONS

Inherited hyperbilirubinemias are the metabolic disorders, mostly manifested in children; therefore their early diagnosis and adequate therapy is of great interest. Currently, there are known all genes responsible for the protein defects, resulting in an increased levels of bilirubin circulated in a blood stream. However, thanks to the rarity of some of hyperbilirubinemias, the estimation of the diagnosis still may take years. The importance of the knowledge of such a disorder is especially in avoiding certain pharmacotherapy, whose effect may change a mild condition to a life threating consequences. During other studies, we would like to highlight several recommendation:

- early molecular diagnosis testing should be taken into account already in non-typical jaundice of newborns
- the molecular genetics testing should consider the population specifics and medical history should already include this information
- as all of the proteins associated with inherited hyperbilirubinemias are also very important entities in biotransformation of toxic compounds and drug metabolizing enzymes, molecular genetic testing should be regularly performed before indication specific pharmacotherapy. Namely in a treatment of oncologic patients or in young adolescents taking hormonal contraceptives.

- genetic counselling in enclosed population with the risk of founder effect mutations about the increased risk of transmission of recessive disorders in consanguineous marriages.

Genetic and biochemical part of our study of inherited hyperbilirubinemias raised following questions to be study:

- what factors, except the genetic ones are affecting the excretion of coproporphyrin isomers?
- what is the cause of inverted ratio of excreted coproporphyrin isomers in patients with DJS and RS?
- in case of Dubin-Johnson syndrome, what is the importance of localization of the mutation in *ABCC2* on excretion of coproporphyrin isomers?

LIST OF ORIGINAL PUBLICATIONS

Publications related to the PhD thesis

The PhD thesis is based on two papers published in journals with impact factor

1. **Slachtova L,** Kemlink D, Martasek P, Kabicek P. *Does Bilirubin Level Correspond to Interaction of c.-3279T>G and A(TA)7TAA Variants in UGT1A1 Gene?* Cell Mol Biol 2009, 55: 95-98 (IF 2009 = 1, 154)
2. **Slachtova L,** Seda O, Behunova J, Mistrik M, Martasek P. *Genetic and biochemical study of Dual hereditary jaundice: Dubin-Johnson & Gilbert syndrome. Haplotyping and founder effect of deletion in ABCC2* Eur J Hum Gen 2015, paper accepted, (IF 2015 = 4,349)

Publications not related to the PhD thesis, published in journals with IF

Boraska, Franklin, Floyd et al*; A genome-wide association study of anorexia nervosa*, Mol Psychiatry. 2014 Feb 11

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