Identification of the deletions in the *UGT1A1* gene of the patients with Crigler–Najjar syndrome type I from Slovakia

I. Zmetáková,[1] V. Ferák,[1] G. Minárik,[1] A. Ficek,[1] H. Poláková,[2] E. Feráková[1] and Ľ. Kádaši[1, 2]

[1] *Department of Molecular Biology, Faculty of Natural Sciences, Comenius University, Bratislava,Slovakia*
[2] *Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovakia*

Abstract. Crigler–Najjar syndrome type I (CN I) is a rare autosomal recessive disorder due to hepatic dysfunction of uridine diphospho-glucuronosyltransferase (UGT) activity toward bilirubin. Complete inactivation of this enzyme causing CN I lead to accumulation of unconjugated bilirubin in serum and bile. Here we report the results of the molecular characterization of the uridine diphospho-glucuronosyltransferase 1A1 (*UGT1A1*) gene in a consanguineous family of Slovak Roms and an unrelated non-Romany family with CN I. Sequence analysis of *UGT1A1* gene in all four Romany patients showed mutation in exon 4, a deletion of an A at codon 407 (1220delA), not yet described in homozygous status. All analysed patients were homozygous for 1220delA mutation and their 3 healthy sibs were heterozygous. The non-Romany patient was a compound heterozygote for two different deletions, 1220delA and 717-718delAG at codon 239. In the family of his cousin a son was born affected with CN I, who was homozygote for 717-718delAG mutation. His other niece affected with CN II was heterozygote for mutation 717-718delAG but homozygote for TA insertion and enhancer substitution T-3279G. Haplotype analysis suggests that the 1220delA mutation is identical by descent in both families, though they originate from two ethnically different populations (Slovaks *vs*. Roms).

Key words: Crigler–Najjar syndrome type I — Gilbert syndrome — Sequence analysis — *UGT1A1* gene

Introduction

Crigler–Najjar syndrome (CN, MIM 218800) is an inborn error of metabolism resulting from mutations in the uridine diphospho-glucuronosyltransferase 1A1 (UGT1A1, MIM 191740) gene, which encodes the bilirubin uridine diphospho-glucuronosyltransferase (B-UGT). B-UGT is the only physiologically relevant human enzyme that catalyzes the glucuronidation of bilirubin (Bosma et al. 1994). B-UGT belongs to the family of transmembrane proteins, UGTs, located in the endoplasmic reticulum and the nuclear envelope of various cells (Roy Chowdhury et al. 1985; King et al. 2000).

Bilirubin is a toxic metabolite of heme, which is produced in large quantities from the normal turnover of hemoglobin and other hem proteins as cytochromes, catalase, and peroxidase. It is nonpolar and circulates in the blood bound to albumin. Bilirubin is taken up efficiently from plasma by hepatocytes *via* facilitated diffusion. In the endoplasmic reticulum of hepatocytes B-UGT catalyzes the transfer of glucuronosyl moiety from uridine diphospho (UDP)-glucuronic acid to bilirubin, forming bilirubin monoglucuronide and diglucuronide, which are excreted in bile. Bilirubin diglucuronide is the major pigment excreted in bile.

Gong et al. (2001) completed the description of the *UGT1* gene complex locus which spans 218 kb. The locus was shown to encode a family of UDP-glucuronosyltransferases including two bilirubin transferase (*UGT1A1* gene), three bilirubin-like, and eight phenol transferase isozymes. In the 5′ region of the locus 13 different exons 1 are located, each with an upstream specific promoter, while the 3′ region contains four common exons. Exons 1 are linked to four common exons to allow for the independent transcriptional initiation to generate overlapping primary transcripts. Each exon 1 encodes the unique amino-terminus of a specific transferase, which is responsible for the substrate selection. The four common exons (2–5) encode the common car-

Correspondence to: Iveta Zmetáková, Department of Molecular Biology, Faculty of Natural Sciences, Comenius University, Mlynská dolina B2-210, 842 15 Bratislava, Slovakia
E-mail: izmetakova@centrum.sk

boxyl-terminus of each isoform that binds the sugar donor substrate UDP-glucuronic acid. First of the 13 unique exons 1 to the four common exons (2–5) of the *UGT1* gene complex is the unique exon 1 of the *UGT1A1* gene.

CN is characterized by severe nonhemolytic unconjugated hyperbilirubinemia and it has been classified, according to the severity of the disease, into two types (Arias 1962).

CN type I (CN I) (Crigler and Najjar 1952) is a rare autosomal recessive disorder due to total absence of hepatic B-UGT activity (UGT, E.C. 2.4.1.17) toward bilirubin. Since birth have the patients with CN I persistent elevation of unconjugated bilirubin in serum and bile, usually above 340 μmol/l level, which may cause later bilirubin encephalopathy (kernicterus – bilirubin forms deposits in the central nervous system leading to lethal neurotoxicity) and death in infancy or childhood. Phototherapy is the preferred long-term treatment for CN I patients. If serum bilirubin level cannot be kept below 450 to 500 μmol/l range, liver transplantation is currently the only definitive therapy.

For CN type II (CN II, MIM 606785) both autosomal recessive and dominant inheritance with variable penetrance have been reported (Arias 1962). B-UGT activity is usually less than 10% of normal. Type II patients have serum bilirubin level in the 120–340 μmol/l range, and respond to phenobarbital treatment (Seppen et al. 1994) by induction of the residual B-UGT activity with consequent reduction of serum bilirubin level. Clinically, this form of icterus is not as acute as type I, and it is rarely associated with kernicterus.

Although the incidence of CNs is not known precisely, it appears to be less than one in a million live births (Van der Veere et al. 1996).

The third inherited disorder of bilirubin glucuronidation, Gilbert syndrome (GS, MIM 143500), is one of the most common inherited disorders in humans, and represents the mildest form of inherited unconjugated hyperbilirubinemia with prevalence approximately 10–13% in Caucasians. Serum bilirubin level fluctuates from normal to 85 μmol/l. GS is associated with structurally normal *UGT1A1* gene encoding region, but with a variant type of promoter and enhancer area upstream to the coding sequence. While the normal TATAA element has the sequence $A(TA)_6TAA$, the Gilbert-type promoter has most often the sequence $A(TA)_7TAA$ (Bosma et al. 1995). The most frequent mutation in enhancer area is substitution T-3279G (Sugatani et al. 2002). These mutation, ($A(TA)_7TAA$ and T-3279G), together reduced the expression of the structurally normal enzyme to about 30% of the normal value.

In the human *UGT1A1* gene 77 defects have been described so far, that are associated with defective bilirubin glucuronidation (Kadakol et al. 2000; Servedio et al. 2005).

Here we present the results of mutation analysis of the *UGT1A1* gene in six CN I and one CN II patient from Slovakia.

Subjects and Methods

We investigated four patients (sibs) with CN I, aged 5 to 17 years, born to consanguineous (double-first cousin) parents from an endogamous community of Roms (Gypsies), and an unrelated non-Romany family with two patients affected with CN I and one patient with CN II. A pedigree of the Romany family is shown in Fig. 1. All affected sibs have jaundice and persistent nonhemolytic severe unconjugated hyperbilirubinemia with neurological disturbances. The first signs of jaundice were observed on 2nd day after birth. Unconjugated hyperbilirubinemia persisted despite repeated phenobarbital therapy. Limited reduction of serum bilirubin level was observed with whole body phototherapy (12 h/day), which is used continuously in all patients. During the last

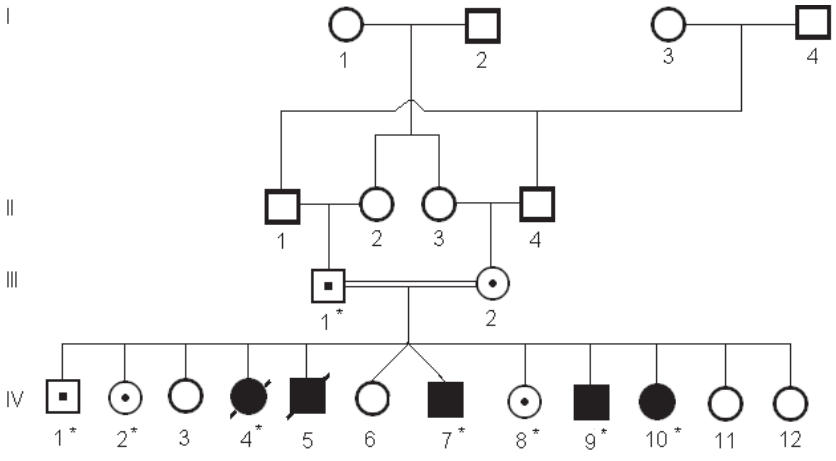

Figure 1. Pedigree of the CN I family. * family members whose DNA was available and analyzed.

Table 1. Primers used for polymerase chain reaction (PCR) and sequencing of the *UGT1A1* gene

	Name of primers	5′ sequence 3′	Size of PCR products (bp)
Enhancer	CN – EF	TCT TCA CCT CCT CCT TAT TC	476
	CN – ER	GGG CAC AGA AAA TTC AGA	
TATA box	CN – TBF	GCT CCA CCT TCT TTA TCT CTG	266
	CN – TBR	TCA ACA GTA TCT TCC CAG CA	
Exon 1	CN – 1F	GGT GTA TCG ATT GGT TTT TGC	1013
	CN – 1R	GGG CTA GTT AAT CGA TCC AAA	
Exon 2	CN – 2F	CAA ACA CGC ATG CCT TTA ATC	363
	CN – 2R	GGG AAA AGC CAA ATC TAA GGT	
Exon 3	CN – 3F	TCA GAG GAC CCC TGT TTT C	450
	CN – 3R	GGG AGG ATG TCA GCA GTT AC	
Exon 4	CN – 4F	GGC ATG TGA GTA ACA CTG AGT C	522
	CN – 4R	AGC CTA GGT GAC AGA GCA AGA	
Exon 5	CN – 5F	GCA GCC ATG AGC ATA AAG AG	509
	CN – 5R	AAG CAG TCT GGG GCT GAT T	

3 years, the serum bilirubin level of the patients ranged between 370–480 μmol/l.

The unrelated non-Romany patient aged 28 years affected with CN I was born to non-consanguineous parents. His serum bilirubin level varied between 560–610 μmol/l despite phototherapy and phenobarbital treatment. He received a liver transplant at the age of 19 years. At the time of liver transplantation he had developed mild form of brain damage. After transplantation, his serum bilirubin levels rapidly decreased to normal. The niece of this patient with serum bilirubin level of 295 μmol/l and the family of his cousin, with CN I affected son, were also investigated. The affection of the two years old niece was clinically described as CN II.

Genomic DNA was extracted from EDTA anticoagulated peripheral blood by phenol-chlorophorm extraction. All five exons of the *UGT1A1* gene involving their flanking intronic regions, the promoter area, and the phenobarbital-response enhancer module (PBREM) were amplified by polymerase chain reaction (PCR). It seems like the oligonucleotide primers were designed by themselves (Tab. 1).

A total of 25 μl reaction volume contained 100 ng human genomic DNA, 2.5 μl of 10 × PCR buffer (10 mmol/l Tris-Cl, 1.5 mmol/l MgCl2, 50 mmol/l KCl, 0.17 mg/ml BSA, 0.1% Triton-X 100), 200 μmol/l of each dNTPs (Invitrogene), 0.2 μmol/l primers for exons 1, 2, 5 and enhancer area, but 0.12 μmol/l primers for exons 3, 4 and promoter of the UGT1A1 gene (VBC Genomics, Wien, Austria) and 0.5 unit of Taq polymerase (Life Technologies, Wien, Austria).

Thirty cycles of amplification were performed in thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). Conditions for PCR were: initial denaturation at 94° C for 4 min, followed by 30 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 2 min. A final extension step was at 72°C for 7 min.

PCR products were sequenced by dye terminator sequencing on an Applied Biosystems model 310 Genetic Analyser and model 3100-Avant Genetic Analyser (Foster City, USA).

Results and Discussion

Out of 12 children, born to consanguineous parents, we have examined 4 patients with CN I, their 3 unaffected sibs, and their father. Sequence analysis of exons 1–5 and promoter and enhancer area of *UGT1A1* gene in all 4 patients showed a mutation in exon 4, a deletion of an A at codon 407 (1220delA), resulting in a frameshift and creating downstream a stop codon at position 410, thus resulting in the production of a shortened functionless protein (Fig. 2). Deletion 1220delA has been previously described by Kadakol et al. (2000, 2001) in 2 girls who presented with severe neonatal hyperbilirubinemia resulting in kernicterus. These patients were heterozygous for this deletion, and at same time, also heterozygous for the *UGT1A1* promoter insertion on the structurally normal *UGT1A1* allele. All four patients from the Romany kindred were homozygous for 1220delA mutation in exon 4 and for substitution T-3279G in enhancer of the *UGT1A1* gene and all 3 healthy sibs and their father were heterozygous for these two mutations. No other change in DNA sequence was observed in other exons as well as in the promoter area. This is the first description of homozygosity for the 1220delA mutation. The homozygosity is a consequence of consanguinity among the parents (double-first cousin).

Sequence analysis of *UGT1A1* gene of the other CN I patient of Slovak non-Romany origin revealed that he was

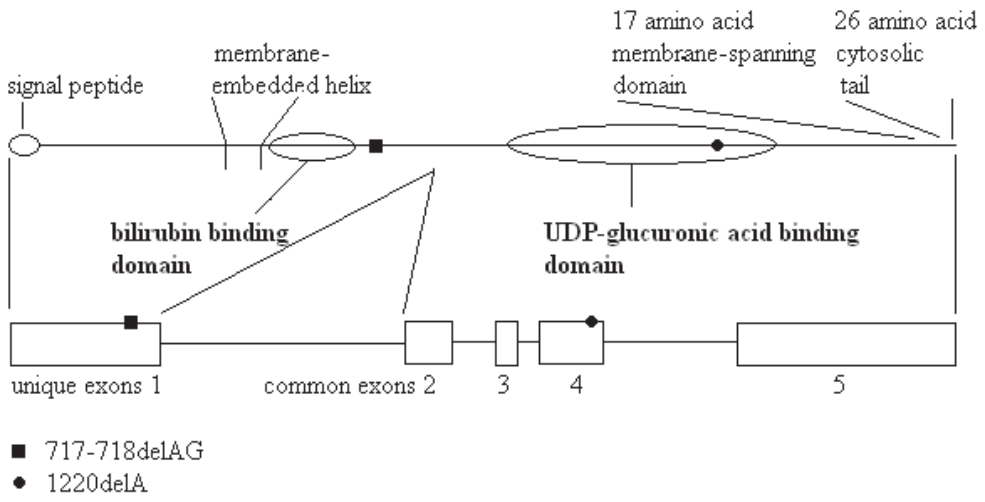

Figure 2. Schematic illustration of the *UGT1A1* gene and of the encoded protein B-UGT with indicated positions of the identified mutations and of the functional domains.

heterozygous for 2 different deletions in exons 1 and 4, and at the same time, heterozygous for the *UGT1A1* promoter insertion A(TA)$_6$TAA/A(TA)$_7$TAA and homozygote for enhancer substitution T-3279G. In the exon 4 the 1220delA mutation was present, and in exon 1 another deletion of 2bp (AG) at codon 239 (717-718delAG). The translational effect of this mutation caused a premature truncation of the B-UGT protein due to frameshift, and created a stop codon TAA at position 247 (Fig. 2). The mutation 717-718delAG was previously described both in patients with CN I in homozygous state (Kadakol et al. 2000) and in patients with CN II in heterozygous state, together with two different missense mutations, also in heterozygous state (Iolascon et al. 2000; Kadakol et al. 2000; Maruo et al. 2003). However, in our study, this is the first description of heterozygozity for two different deletions of the *UGT1A1* gene in a CN I patient. Our results confirm that the promoter variant (TA)$_7$ is in cis phase with 717-718delAG (Iolascon et al. 2000), and the (TA)$_6$ variant is in cis with 1220delA mutation (Kadakol et al. 2001). The CN II affected niece of our CN I non-Romany patient was heterozygote for deletion 717-718delAG and homozygote for promoter insertion and enhancer substitution T-3279G. The two months old son of the cousin of the non-Romany CN I patient was clinically diagnosed as CN I. He was homozygote for each of mutations 717-718delAG, A(TA)$_7$TAA, and T-3279G. His non-consanguineous parents were found heterozygous for this deletion. The father was homozygote also for both A(TA)$_7$TAA and T-3279G mutations, and the mother was homozygote for T-3279G, but heterozygote for promoter insertion.

Phenobarbital-response enhancer module (PBREM) of the *UGT1A1* gene lies 3 kb upstream the TATA box and contains 6 different single nucleotide polymorphisms (Innocenti et al. 2002; Sugatani et al. 2002). Sequencing of the PBREM and TA variants in the promoter region in our CN I patients with 1220delA mutation revealed a very rare haplotype (ATGCAG at base positions -3440, -3401, -3279, -3177, -3175, -3156). Its frequency in Caucasions was estimated 0.02 by Innocenti et al. (2002). This finding suggests that 1220delA mutation in non-Romany patient has common origin with that observed in the Romany family, in spite of the fact that both families originate from ethnically different populations (Roms *vs.* non-Romany Slovaks).

In humans, CN I is a rare autosomal recessive disorder, which was first described in 1952, and is often found in children of consanguineous parents. Here we described for the first time the *UGT1A1* gene mutations in Slovak patients with CN I. Four Romany CN I patients, siblings from a consanguineous family, were all homozygous for 1220delA, and their three healthy sibs and father were heterozygous for this mutation. One non-Romany Slovak patient was a compound heterozygote for two different deletions – 1220delA and 717-718delAG – and also heterozygote for a TA promoter variant and homozygote for enhancer substitution T-3279G. The son of his cousin was homozygote for deletion 717-718delAG, and also for TA promoter insertion and substitution T-3279G in the enhancer area. The niece of this non-Romany CN I patient with CN II was heterozygous for 717-718delAG mutation and homozygote for mutations in enhancer and promoter area too.

Coinheritance of the promoter and enhancer mutations with a mutation (717-718delAG) in the coding region of the *UGT1A1* gene is responsible for a CN II phenotype. Gilbert´s mutation (A(TA)$_7$TAA and T-3279G) can modify the *UGT1A1* mutation phenotype, by reducing the expression of the *UGT1A1* gene and so play a role in enhancing the pathogenic effect of the heterozygous coding mutation.

Acknowledgements. We would like to thank Prof. Viera Kupčová, M. D. Eduard Šiška, and M. D. Jana Kosnáčová for their kind cooperation and help by providing the blood samples, and to Ildikó Szomolay for her technical assistance. This project was partially supported by Grant of Comenius University (grants No. 148/2003, 250/2004), and by grant of Ministry of Health No. 2005/4-DF-NsPBA-02.

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Final version accepted: November 30, 2007

New Books

Methods in Membrane Lipids

Alex M. Dopico (*Editor*)
2007, 621 pages, Hardcover
Price: 149 $
Humana Press Inc., Totowa, New Jersey *(www.humanapress.com)*
Series: Methods in Molecular Biology, Volume 400
eISBN: 978-1-59745-519-0
ISSN: 1064-3745

Biological membranes form the interface between the cell and its environment; they compartmentalize eukaryotic cells and are key players in cellular homeostasis and metabolic-energy transduction. Membrane lipids are not only passive components of the bilayer forming the permeability barrier but are increasingly being recognized as versatile and dynamic regulators of numerous cellular processes that range from cell growth, development and survival to intracellular signaling, cargo sorting and membrane trafficking. It is known that cellular membranes contain many more lipid species than are needed to form a bilayer. Importantly, lipid distribution in the membranes is non-random between different biological membranes as well as between two leaflets of membranes. In addition, membrane lipids are laterally organized in the bilayer plane, which leads to the existence of specific lipid domains. As a consequence of this complicated and elaborated lipid distribution sophisticated new tools are needed to study membrane lipids and their role in cell function.

The present issue of the Methods in Molecular Biology series, entitled „Methods in Membrane Lipids" is dedicated to the presentation of methodologies developed by both experimentalists and theoreticians to study structure and function of membrane lipids. The book is organized around individual biological problems. Thus, the book sequentially presents methods to study i) lipid distribution, structure, and lipid-lipid interactions; ii) lipid phases; iii) mono- and bilayer lipid curvature and stress; iv) lipid domains; and v) membrane lipid-protein and lipid-drug interactions. Two introductory chapters included in this book provide a brief overview of the diversity of membrane lipids and their organization. Most experimental chapters are written in the typical format of the Methods in Molecular Biology series; they contain an introduction describing biological problem and the principles of the technique, followed by Materials and Methods sections containing a "cookbook" for a particular experimental technique. Each chapter contains also Notes section in which experimental difficulties, pitfalls and the ways to circumvent them are described. I greatly acknowledge an extensive reference list at the end of every chapter.

Total of 38 different approaches used in membrane lipid research are covered in the book. Described methodologies vary greatly from lab bench experimentation (e.g. methods to define lipid rafts and caveolae) and approaches requiring rather standard equipment (fluorescent assays to measure fatty acid binding and transport, methods to measure lipid diffusion in the membranes, determination of sterol oxidation) to the methods requiring sophisticated and specialized experimental setup (Raman scattering microscopy, X-ray scattering, atomic force microscopy, optical dynamometry). Chapters describing various computer simulations and theoretical models (e.g. analysis of biomembrane NMR data, statistical thermodynamics to characterize phospholipids interactions in membranes) require serious knowledge of higher mathematics. Saying that, it is not the book for beginners, but thanks to the wide range of topics, every reader interested in membrane lipid research, especially those studying membrane organization, will find several interesting chapters in it. Every such a wide-range book reflects the preferences of the editor for individual methods and approaches. Nevertheless, in the book entitled "Methods in Membrane Lipids" the whole area of application of mass spectroscopy to lipid analysis should not be omitted. I certainly miss this methodology in the book.

Taken together, volume 400 of the Methods in Molecular Biology series, entitled "Methods in Membrane Lipids", presents a broad spectrum of mostly biophysical methods to study membrane lipid distribution, behavior and organization as well as methods to study interactions among lipids, membrane proteins and drugs. Mostly the biophysics and biochemists involved in membrane research will find the book especially useful. The book could also serve as a reference for the scientists in other areas to recognize the potential (and limitations) of modern biophysical methods of membrane research.

Peter Griač