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Case Report

Identification of a novel mutation in UDP-glucuronosyltransferase (*UGT1A1*) gene in a child with neonatal unconjugated hyperbilirubinemia

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ABSTRACT

Genetic alterations of the *UGT1A1* gene result in Crigler–Najjar (CNS) and Gilbert's (GS)-Syndromes, two autosomal recessive conditions characterized by non-hemolytic unconjugated hyperbilirubinemia. While GS is characterized by mild hyperbilirubinemia, CNS is classified as follows: type I (CNS-I), often associated with irreversible neurological damage due to total deficiency of the UGT1A1 enzyme activity, and type II (CNS-II) where a minimal level of UGT1A1 enzyme activity is maintained. In this context, differential diagnosis of CNS forms needs to be supported by clinical molecular laboratory, in order to correlate biochemical findings to specific genetic mutations.

Our paper describes in detail the peculiar clinical feature found in a child with severe neonatal unconjugated hyperbilirubinemia, where DNA analysis showed a new compound heterozygosis determined by two mutations, a known (c.508_510delTTC) and a novel mutation (c.1099C>T) giving a genotype compatible with clinical picture of CNS-II.

This novel genotype extends the spectrum of known *UGT1A1* mutations, which, in our opinion, could be higher than that currently reported in the literature. Finally, genetic analysis may also be helpful for patients' management.

Introduction

Bilirubin, final product of the heme's degradation, is efficiently eliminated from the body through the biliary tract. Due to its low water solubility, the conversion into the bile canaliculus to a water-soluble molecule represents a fundamental step in the excretion of the bilirubin. For this purpose, bilirubin is conjugated with glucuronic acid by the isoenzyme uridine-5′-diphosphate glucuronosyltransferase isoform 1A1 (UGT1A1), encoded by *UGT1A1* gene. Genetic alterations of the *UGT1A1* gene result in Crigler–Najjar (CNS) and Gilbert's (GS) Syndromes, two autosomal recessive conditions characterized by non-hemolytic unconjugated hyperbilirubinemia. GS is characterized by a reduction (approximately 70%) of *UGT1A1* gene expression with serum total bilirubin concentration (STBC) that rarely exceeds 85 μmol/L [1]. According to STBC, CNS is classified in two types: type I (CNS-I) and type II (CNS-II). In CNS-I, often associated with irreversible neurological damage, mutations in *UGT1A1* gene determine total deficiency of the UGT1A1 enzyme activity, resulting in high levels of STBCs (340–765 μmol/L). On the contrary, in CNS-II patients, irreversible neurological damage, due to enhanced bilirubin load, can occur only in particular conditions (as trauma or sepsis), since UGT1A1 enzyme activity is variable (with a range of STBC within 102–340 μmol/L). For the above mentioned reasons, early identification of these patients could be helpful in preventing irreversible brain damages by close surveillance and additional treatments [2].

In this paper we present the case of a child with severe neonatal unconjugated hyperbilirubinemia, where DNA analysis (by direct sequencing of the entire coding sequence and promoter of the *UGT1A1* gene) showed a new compound heterozygosis determined by two mutations, one known [3] while the second one discovered as a novel variant: this genotype was associated to clinical picture of CNS-II.

Case report

An Italian male (with birth weight of 3320 g) was born at full term from non-consanguineous parents. At medical examination he resulted as normal, while biochemical tests revealed severe unconjugated hyperbilirubinemia. At second day of life, the levels of STBC were 357 μmol/L; for this reason, the child was treated with phototherapy for 15 days. Fig. 1 shows the trend of concentrations of serum total and unconjugated bilirubin. Serological tests for hepatocellular integrity, such as ALT (alanine aminotransferase), AST (aspartate aminotransferase), ALP (alkaline phosphatase) activities, serum albumin and total

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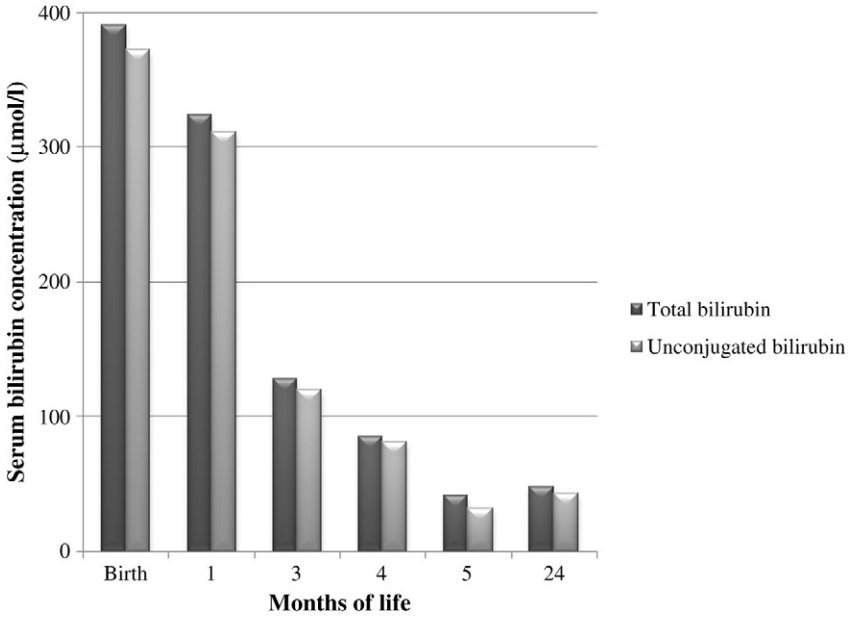

Fig. 1. Trend of serum total and unconjugated bilirubin concentrations of the patient in the first 24 months of life. At about two years old, patient shows a total bilirubin of 50 μmol/L. We believe that this value is not the baseline value but rather a transitional likely to increase with age.

protein concentrations resulted within normal range. Furthermore, viral neonatal infections, red blood cell (RBC) enzyme abnormality, hematoma, rash, or diseases of his central nervous system were excluded. Neither neonatal jaundice nor increased STBC, or congenital abnormalities, mental retardation, neonatal death, hepatic disease and neurological disorders were present in his parents. Although clinical examination of neonate did not show any clinical sign of CNS, the above biochemical findings suggested deeper laboratory investigations of the *UGT1A1* gene.

Materials and methods

After informed consent statement was obtained, blood samples from proband and his parents were collected. Genomic DNA was extracted by an automatic DNA device (Maxwell^{M} 16, Promega, Madison, USA) and DNA concentration and quality were determined spectrophotometrically (NanoPhotometer™, Implen, München Germany, http://www.implen.de).

Primers used for PCR and direct sequencing of *UGT1A1* gene are reported in Table 1. PCR reactions were set up in 25 μL obtained by adding: 12.5 μL of MasterMix 2× [Promega, Madison, USA, http://www.promega.com, containing Buffer (pH = 8.5), dNTPs (400 μM), Taq Polymerase (50 units/μL) and Mg^{2+} (3 mM)], 3–4 μL of genomic DNA as template (about 120–180 ng), 0.5 μL of both primers (200 nM) and ultrapure H_2O until reaching the final volume.

Table 1
Primers used for amplification of the coding and promoter's regions of the *UGT1A1* gene.

UGT1A1 region	Primers	Sequence (5′→3′)	Ta
Promoter	UGT1A1-prom-F	5′-GACGTTCTGGAAGTACTTTGC-3′	56°
	UGT1A1-prom-R	5′-CCAAGCATGCTCAGCCAG-3′	
Exon 1	UGT1A1-AF	5′-AGGAGCAAAGGCGCCATGGC-3′	58°
	UGT1A1-AR	5′-TGCAGTAAGTGGGAACAGCC-3′	
	UGT1A1-F1	5′-TTGTCTGGCTGTTCCCACTT-3′	
	UGT1A1-BR	5′-CGCATGTAAAAGTCCCAGTCC-3′	
Exon 2	UGT1A1-F2	5′-ATTCTGTAAGCAGGAACCCT-3′	58°
	UGT1A1-R2	5′-TAATAGTTGGGAAGTGCCAG-3′	
Exon 3	UGT1A1-F3	5′-ATAGTTCTGCATCCACTTGTT-3′	58°
	UGT1A1-R3	5′-TGTTACTCACATGCCCTTGC-3′	
Exon 4	UGT1A1-F4	5′-GGCTTAAGCACAGCTATTCT-3′	58°
	UGT1A1-R4	5′-CATGAATGCCATGACCAAAGT-3′	
Exon 5	UGT1A1-F5	5′-TGCAGTTAGCCATGCTTGTG-3′	58°
	UGT1A1-R5	5′-TTTGGAAATGACTAGGGAATGG-3′	

The program consisted of 35 cycles with the following conditions: denaturation at 95 °C for 2′, annealing for 30″ (56°–58 °C), and extension at 72 °C for 60″. A negative reaction was run in order to avoid cross-contamination of reagents used for the set-up of our PCR procedure. After checking of the amplification product by gel agarose electrophoresis, the amplicons were purified using Amicon® Ultra (Millipore, Bedford, MA, USA) and sequenced in both forward and reverse directions using the same PCR primers and the ABI BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystem, Foster City, CA USA, http://www.appliedbiosystems.com/absite/us/en/home.html) on ABI Prism 3100 Genetic Analyzer. Finally, sequences were aligned to the NG_002601 reference sequence, using the SeqScape® Software v2.5 (Applied Biosystem, Foster City, CA USA). In each sequencing run, we analyzed: 1) as positive internal quality control, DNA from a patient previously clinically and genetically classified as affected by Crigler Najjar type II, 2) the pGEM®-3Zf(+) Vector (Applied Biosystem, Foster City, CA, USA) in order to evaluate the performance of direct sequencing. A base call accuracy of 99% (Q20) was used by default for sequence analysis and interpretation being it considered as the most commonly used method for high quality base assignment [4].

Results

The direct sequencing of entire *UGT1A1* gene showed that proband was compound heterozygote for the known mutation (c.508_510delTTC) [3], and the novel c.1099C>T variant in exon 4. To confirm these findings, the entire process (DNA extraction, PCR and sequencing) was repeated using a second independent blood sample of our patient. The c.508_510delTTC determines a deletion of the phenylalanine (F) at position 170, abolishing a conserved diphenylalanine site [3]. The transition of C to T at nucleotide 1099 (exon 4) predicts the substitution of argynine (R) with cysteine (C) at codon 367 of the enzyme. Since this substitution was not reported in the literature, we considered it as a possible novel mutation, due to its absence in 100 additional healthy individuals analyzed. Finally, parents of proband were carriers of the deletion and novel mutation, respectively, confirming the inheritance and the allelic

distribution in trans of the two mutations. Screening for the TA duplication in the *UGT1A1* gene promoter of the proband and his parents showed a wild type genotype (6TA/6TA), excluding the Gilbert syndrome condition.

Discussion

Genetic alterations causing absence, or severe reduction of UGT1A1 activity and resulting respectively in CNS-I and CNS-II syndromes [5] can be located in any of the five exons of the *UGT1A1* gene. Mutations causing stop codon, frame-shift, or deletion of critical amino acids, as disruption of the hydrophobicity within a stretch of 20 aromatic/hydrophobic amino acids in the amino-terminal domain of UGT1A1 enzyme (encoded by exon-1) [6], drastically reduce enzyme activity and result in CNS-I. In the case of single amino acid substitutions, the phenotype cannot always be predicted from the mutation alone; only substitution of single amino acid residues within certain micro-regions of UGT1A1 enzyme (such as highly conserved region, amino acids 298–315) [7] can lead to complete, or partial loss of enzyme activity, resulting in an alternative feature of CNS-I.

In CNS-II, mutation always determines the substitution of a single amino acid, reducing enzyme activity, but does not abolish it [7]. This explains the milder phenotype observed in CNS-II and the inducibility of the residual enzyme activity by phenobarbital administration.

In this paper, we report the case of a newborn suffering from hyperbilirubinemia and resulted compound heterozygote for two *UGT1A1* gene mutations. Despite the proband presented with severe neonatal hyperbilirubinemia, no kernicterus was developed due to the rapid good response to phenobarbital and UV treatments. Based on the history, along with biochemical and genetics findings, clinical diagnosis of CNS-II was made. His parents (father and mother carried the c.1099C>T and the c.508_510delTTC mutations, respectively), did not present any sign of hyperbilirubinemia according with autosomal recessive inheritance of this metabolic disorder.

Deletion of a phenylalanine at codon 170, found in our patient, abolishes a conserved diphenylalanine site inserted in a highly conserved strikingly hydrophobic region between amino acids 161 and 180 (amino-terminal region of the UGT1A1 enzyme) and accounting for 80% of the bilirubin transferase activity. Therefore, this mutation could be considered highly deleterious, because it drastically reduces the enzyme activity. To this regard, Rosatelli et al. [8] reported that, homozygous state, c.508_510delTTC determines a CNS-I phenotype. Contrasting with data by Rosatelli et al., who found this mutation in Sardinia (suggesting a possible founder effect), the family members of our patient did not report any Sardinian ancestor: for this reason, we believe that this mutation can also be present in other Italian regions.

In our patient, the c.508_510delTTC mutation is in compound heterozygous with a novel *UGT1A1* mutation (c.1099C>T) which causes an amino acid substitution (p.R367C) in the catalytic core of the UGT1A1 protein. At the same codon, another mutation (p.R367G) has previously been reported [9] in association with GS. The phenotype associated with our patient (CNS-II) confirms that this amino acid does not play a critical role for the enzyme activity, despite the substitution of the arginine to cysteine inserts a highly reactive thiol group. These findings suggest that the coexistence of the deletion (c.508_510delTTC) and the c.1099C>T mutation does not determine the complete abolition of UGT1A1 enzyme function, but results only in a mild phenotype characterized at variable levels of STBC, with high concentrations at birth followed by a progressive declining over time up to reach values slightly above the normal ranges. The clinical phenotype observed was considered compatible with CNS-II: in fact, patients with CNS-II may have STBCs ranging from mild to high levels, sometimes triggered by conditions such as the presence of stress, infection, or liver failure or during the neonatal period.

In conclusion, we believe that *UGT1A1* gene analysis should be performed in all cases where suspect of genetic dependent-unconjugated hyperbilirubinemia is present. In these cases, the absence of mutations in *UGT1A1* gene may lead clinicians to investigate other metabolic disorders where hyperbilirubinemia may be associated to hemolysis (such as erythrocyte enzymopathies [10,11]). In addition, the description of patients with peculiar genotypes could explain the phenotype prediction, helping clinicians in the patients' management.

Finally, in our opinion, the prescription of *UGT1A1* genetic test in patients at risk, could improve in the future, epidemiological data regarding frequency of CNS mutations in Italy.

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