Role of a homozygous A(TA)$_7$TAA promoter polymorphism and an exon 1 heterozygous frameshift mutation *UGT1A1* in Crigler-Najjar syndrome type II in a Thai neonate

P. Nilyanimit[1], A. Krasaelap[1], M. Foonoi[2], V. Chongsrisawat[2] and Y. Poovorawan[1]

[1]Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
[2]Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Corresponding author: Y. Poovorawan
E-mail: Yong.P@chula.ac.th

ABSTRACT. Crigler-Najjar syndrome is a rare autosomal recessive disease caused by mutations in the *UGT1A1* gene. These mutations result in the deficiency of UGT1A1, a hepatic enzyme essential for bilirubin conjugation. This report describes the case of a 4-month-old boy with the cardinal symptoms of Crigler-Najjar syndrome type II. Molecular genetic analysis showed a homozygous *UGT1A1* promoter mutation [A(TA)$_7$TAA] and a heterozygous insertion of 1 adenosine nucleotide between positions 353 and 354 in exon 1 of *UGT1A1* that caused a frameshift with a premature stop codon.

Key words: Uridine glucuronosyltransferase 1; Crigler-Najjar; *UGT1A1*; Phototherapy

INTRODUCTION

Bilirubin is a toxic product of the breakdown of heme-containing proteins and is derived mostly as a by-product of hemoglobin metabolism (Tenhuhen et al., 1969). In order for hydrophobic bilirubin to be detoxified and excreted, it has to be glucoronized by hepatic uridine 5'-diphosphoglucoronosyltransferase-1 (UGT-1). This is achieved mainly by the UGT1A1 isozyme (Ritter et al., 1991). This enzyme is encoded by the *UGT1A1* gene, which is located on chromosome 2q37 and consists of 5 consecutive exons (Kadakol et al., 2000). Autosomal recessive inheritance of variants in *UGT1A1*, either in an exon or the promoter, may result in UGT1A1 enzyme deficiency and the impairment of bilirubin conjugation in hereditary unconjugated hyperbilirubinemia syndromes, type I and II Crigler-Najjar syndrome, and Gilbert's syndrome.

Type I and II Crigler-Najjar syndrome and Gilbert's syndrome have been classified based on bilirubin levels, severity of symptoms, and response to the administration of UGT1A1 inducers, such as phenobarbital (Seppen et al., 1994). The clinical manifestations of these syndromes range from asymptomatic, episodic, mild hyperbilirubinemia in Gilbert's syndrome to kernicterus, irreversible brain damage, and death in Crigler-Najjar syndrome type I.

As originally reported by Crigler and Najjar in 1952, Crigler-Najjar syndrome type I is characterized by an almost complete loss of UGT1A1 activity resulting in life-threatening hyperbilirubinemia in the range of 20-45 mg/dL, and a lack of response to phenobarbital (Labrune et al., 1989). With Crigler-Najjar syndrome type II, partial loss of UGT1A1 activity causes less hyperbilirubinemia (6-20 mg/dL) resulting in better outcomes, with a fair response to phenobarbital characterized by a 30% decrease in serum bilirubin. The mildest and most common phenotype associated with *UGT1A1* mutations is Gilbert's syndrome. The serum bilirubin levels in patients with Gilbert's syndrome typically fluctuate between 1-6 mg/dL (Labrune et al., 1989).

To date, more than 100 mutations in the *UGT1A1* gene have been reported in the Human Gene Mutation Database (www.hgmd.cf.ac.uk). The most common variant in Caucasians is the presence of an additional TA nucleotide pair in the promoter region, $A(TA)_7TAA$, rather than the normal $A(TA)_6TAA$ sequence. This homozygous variant TATAA element reduces the expression of the structural enzyme as observed in patients with Gilbert's syndrome (Bosma et al., 1995). However, the more common variant in East Asian populations is an intra-exon missense mutation, c.211G>A (Akaba et al., 1998). In addition, some studies have reported that patients who were homozygous for the $A(TA)_7TAA$ allele were also homozygous for another mutation, c.-3279T>G. This variant was found to be one of the risk factors for Gilbert's syndrome or neonatal jaundice in Malays (Ichiro et al., 2010). Here we report the case of a patient with Crigler-Najjar syndrome type II caused by the homozygous $A(TA)_7TAA$ mutation with a heterozygous single adenosine nucleotide insertion between positions 353 and 354 (353_354insA) in exon 1, leading to a frameshift.

CASE REPORT

A 4-month-old Thai male presented jaundice from the age of 1 month. He was the first child of non-consanguineous parents and was born full term by vaginal delivery, with a birth weight of 3140 g. There was no history of jaundice or any liver diseases in the family.

The patient appeared normal at birth; however, he developed progressive jaundice without fever, gastrointestinal, or neurological symptoms from 1 month of age. His initial laboratory results showed peak total bilirubin at 27.7 mg/dL. Thyroid function was normal. The mother's and patient's blood groups were both B-positive. Reticulocyte count and glucose-6-phosphate dehydrogenase levels were normal. His abdominal ultrasonography showed no evidence of bile duct obstruction or abnormal liver echogram. With phototherapy and total blood exchange transfusion, total bilirubin was successfully reduced to 14.5 mg/dL. He was then discharged with home phototherapy at night prior to his referral.

At King Chulalongkorn Memorial Hospital, laboratory tests performed on the patient showed unconjugated hyperbilirubinemia with normal liver function tests (Table 1). Complete blood count showed 11.3 mg/dL hemoglobin, 33.9% hematocrit, and a white blood cell (WBC) count of 8900/μL (18% neutrophils, 72% lymphocytes, 3.7% monocytes, 4.7% eosinophils, 0.5% basophils, 511,000/μL platelets). The data suggested mild anemia with no hemolytic phenomena seen on a peripheral blood smear. Liver function tests performed on his parents yielded normal results (Table 1).

Table 1. Laboratory data of the patient obtained at 4 months of age and his parents at King Chulalongkorn Memorial hospital.

Liver function test	Patient	Father	Mother
Total protein (g/dL)	5.7	8.4	8.6
Albumin (g/dL)	4.5	4.9	4.8
Total bilirubin (mg/dL)	28.89	0.3	0.3
Direct bilirubin (mg/dL)	0.5	0.1	0.1
SGOT (U/L)	34	21	21
SGPT (U/L)	33	22	20
ALP (U/L)	241	81	59
GGT (U/L)	31	-	-

SGOT = serum glutamic oxaloacetic transaminase; SGPT = serum glutamic pyruvic transaminase; ALP = alkaline phosphatase; GGT = gamma-glutamyl transferase.

Based on the presentation of unconjugated hyperbilirubinemia symptoms without neurological complications, a diagnosis of Crigler-Najjar syndrome type II was made, despite persistently high bilirubin levels (>20 mg/dL) and a less than 30% reduction in bilirubin levels upon phenobarbital treatment. Blood samples of the patient and his family were collected and subjected to molecular genetic analysis.

MATERIAL AND METHODS

Molecular genetic diagnosis

Blood samples and DNA extraction

The parents were informed about the objective of genetic testing for the disease diagnosis and subsequently gave their written consent. One milliliter each of ethylenediaminetetraacetic acid (EDTA) blood samples was collected from the patient and parents. Peripheral blood mononuclear cells (PBMCs) were separated using a lymphocyte separation medium (Wisent Inc., Canada). The PBMCs were lysed with red cell lysis buffer (1 M Tris-HCl, pH

7.6, 5 M NaCl, and 1 M MgCl$_2$) and subjected to centrifugation. The pellet was resuspended in 1 mL phosphate-buffered saline. Genomic DNA was extracted from 100 µL WBCs by incubation with lysis buffer (10 mM Tris-HCl, pH 8.0, 0.1 M EDTA, pH 8.0, 0.5% sodium dodecyl sulfate, and 20 mg/mL proteinase K) followed by phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation. The resulting DNA pellet was resuspended in 20 µL sterile distilled water and stored at -20°C.

Amplification of the *UGT1A1* promoter and exons

The promoter and 5 exons of *UGT1A1* were amplified using the primer sets described in Table 2. Polymerase chain reaction (PCR) was carried out using a Mastercycler PCR machine (Eppendorf, Germany). The 25-µL reaction mixture contained 200 ng genomic DNA in 1X PCR buffer with 0.5 µL 10 mmol of each primer, 12.5 µL 2.5X PerfectTaq Plus Master-Mix (5 PRIME Inc., Germany), and 10.5 µL sterile water. The PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. The PCR products were purified from residual agarose using the PCRExtract & GelExtract Mini Kits (5 PRIME).

Table 2. PCR primers and fragments.

Position	Primer pairs (5'→3')	Product size (bp)	Annealing temperature (°C)
Promoter	UGT1A1_F: ATTTGAGTATGAAATTCCAGCC	210	55
	UGT1A1_R: CCAAGCATGCTCAGCCAG		
Exon 1	UGT1A1_F1: GTCACGTGACACAGTCAAAC	1043	61
	UGT1A1-2R1: TGGGGCTAGTTAATCGATCC		
Exon 2	UGT1A1-F234: CTCTATCTCAAACACGCATGCC	869	61
	UGT1A1-R2343: ACTAGAAAACAGGGGTCCTCTG		
Exon 3	UGT1A1-F2342: AAACAAGATGCCGGAAGTTGTCC	526	61
	UGT1A1-R3_N: GAGGATGTCAGCAGTTACATCTC		
Exon 4	UGT1A1-F4_N: GTGTTTTCAGAACCTAGATGTGTCC	395	60
	UGT1A1-R4_N: ACAACGCTATTAAATGCTACGTAAATG		
Exon 5	UGT1A1-F5: GAGGATTGTTCATACCACAGG	496	59
	UGT1A1-2R5: TTTTAAAGCACTCTGGGGCTG		

Nucleotide sequencing and analysis of *UGT1A1*

The direct sequencing reaction was performed by First BASE Laboratories Sdn Bhd (Malaysia) according to manufacturer specifications. Nucleotide sequences from direct sequencing were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR, USA) and subsequently subjected to BLAST analysis.

RESULTS

We screened the *UGT1A1* gene for the A(TA)$_7$TAA, c.211G>A, and c.-3279T>G mutations. The patient was found to be homozygous for the A(TA)$_7$TAA promoter variant containing 7 repeats of TA. Upon searching for an intra-exon mutation in *UGT1A1*, a heterozygous insertion of 1 adenosine nucleotide between nucleotides 353 and 354 in exon 1 (353_354insA) was identified (Figure 1). The amino acid lysine in codon 118 was not changed; however, a

frameshift mutation occurred and resulted in 27 abnormal amino acid substitutions (Gly119-Leu145). No other mutations were identified in exons 2-5.
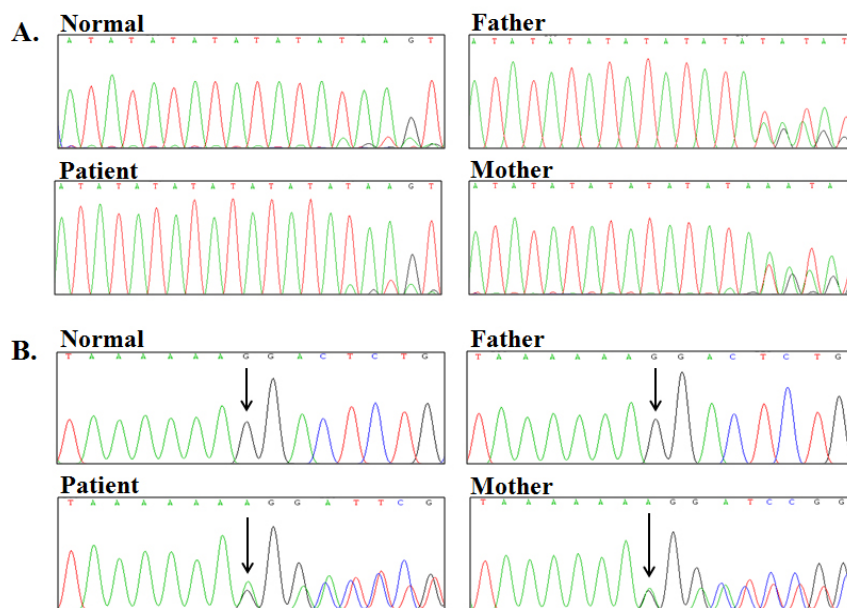

Figure 1. DNA sequence analysis of family members. Nucleotide sequence shows the TA repeat in the promoter (**A**) and the heterozygous insertion A at position 354 in exon 1 (**B**).

The patient's parents had heterozygous promoter mutations, $A(TA)_7TAA/A(TA)_6TAA$, which explained the homozygous $A(TA)_7TAA$ mutation in the patient. Moreover, an exon 1 353_354insA variant, similar to that of the patient, was identified in 1 allele of the mother. The c.211G>A and c.-3279T>G variants, however, were absent in this family.

DISCUSSION

Crigler-Najjar syndrome is normally inherited in an autosomal recessive manner and is caused by homozygous mutations in the *UGT1A1* gene, which is a member of the UGT1A family and contains 5 exons. The unique 1st exon at the 5'-terminus encodes the binding site for bilirubin at the N-terminal and exons 2 to 5 at the 3'-terminus encode the binding site of glucuronic acid at the C-terminal (Aono et al., 1993).

The TATAA element in the promoter region is the binding site for transcription factor IID, which is responsible for the initiation of transcription. The $A(TA)_7TAA$ variant of *UGT1A1*, as in this patient, results in the reduced frequency and accuracy of transcription initiation. The longer the TATAA element, the higher the mean serum bilirubin levels in normal, healthy subjects and in compound heterozygous carriers of Crigler-Najjar syndrome type II (Grosveld et al., 1982; Dierks et al., 1983).

Based on mild clinical manifestation in this patient, the diagnosis of Crigler-Najjar syndrome type II was made despite high bilirubin level (>20 mg/dL) and partial response to

phenobarbital (<30% bilirubin reduction). This patient was heterozygous for an insertion mutation in exon 1 (353_354insA) that was most likely inherited from his mother. The insertion did not alter the amino acid lysine in codon 118; however, it did lead to a frameshift mutation with a premature stop codon. This premature stop codon in exon 1, which encodes the binding site for bilirubin at the N-terminus, results in a truncated protein with impaired catalytic activity on bilirubin. In 2011, Wang et al. reported this insertion mutation in a Uigar boy, who developed clinical jaundice within 3 days of birth. In contrast to the heterozygous insertion mutation found in our patient, the mutation was identified in both alleles of *UGT1A1* in the Uigar boy causing severe Crigler-Najjar syndrome type II symptoms.

The child's mother was heterozygous for both the A(TA)$_7$TAA allele and the insertion allele, whereas the father was heterozygous only for the A(TA)$_7$TAA allele. As neither had ever had jaundice and their bilirubin levels were normal, they were classified as silent carriers. Compared to the mutations in the mother, the presence of only 1 additional A(TA)$_7$TAA allele in the child lead to significant jaundice. This suggests an important role for the homozygous A(TA)$_7$TAA mutation in the development of clinical hyperbilirubinemia in individuals with *UGT1A1* mutations. This homozygous mutation led to the manifestation of the symptoms of Crigler-Najjar type II in this patient.

With home phototherapy and phenobarbital administration, bilirubin levels were gradually reduced to 20 mg/dL. The patient still had jaundice without any neurological impairment. Home phototherapy was discontinued at the age of 11 months, while phenobarbital was continued and has remained the only treatment for Crigler-Najjar syndrome type II in this patient.

In conclusion, we have reported the presentation of Crigler-Najjar syndrome type II in a 4-month-old male patient with congenital severe indirect hyperbilirubinemia without neurological complications who was followed up until 2 years of age. Molecular diagnosis revealed homozygous A(TA)$_7$TAA promoter and heterozygous 353_354insA mutations. Our results suggest an important role for the homozygous A(TA)$_7$TAA mutation in the development of clinical hyperbilirubinemia and Crigler-Najjar syndrome type II in individuals with *UGT1A1* mutations.

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