



Two unrelated patients with rare Crigler-Najjar syndrome type I: two novel mutations and a patient with loss of heterozygosity of *UGT1A1* gene

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Received Sept. 1, 2013; Revision accepted Feb. 9, 2014; Crosschecked Apr. 16, 2014

Abstract: Crigler-Najjar syndrome type I (CN-I) is the most severe type of hereditary unconjugated hyperbilirubinemia. It is caused by homozygous or compound heterozygous mutations of the UDP-glycosyltransferase gene (*UGT1A1*) on chromosome 2q37. Two patients clinically diagnosed with CN-I were examined in this paper. We sequenced five exons and their flanking sequences, specifically the promoter region of *UGT1A1*, of the two patients and their parents. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to determine the *UGT1A1* gene copy number of one patient. In patient A, two mutations, c.239_245delCTGTGCC (p.Pro80HisfsX6; had not been reported previously) and c.1156G>T (p.Val386Phe), were identified. In patient B, we found that this patient had lost heterozygosity of the *UGT1A1* gene by inheriting a deletion of one allele, and had a novel mutation c.1253delT (p.Met418ArgfsX5) in the other allele. In summary, we detected three *UGT1A1* mutations in two CN-I patients: c.239_245delCTGTGCC (p.Pro80HisfsX6), c.1253delT (p.Met418ArgfsX5), and c.1156G>T (p.Val386Phe). The former two mutations are pathogenic; however, the pathogenic mechanism of c.1156G>T (p.Val386Phe) is unknown.

Key words: Crigler-Najjar syndrome type I (CN-I), Hyperbilirubinemia, UDP-glycosyltransferase gene (*UGT1A1*), Mutation, Loss of heterozygosity

doi:10.1631/jzus.B1300233

Document code: A

CLC number: R722

1 Introduction

Crigler-Najjar syndrome type I (CN-I; MIM #218800) is the most severe type of hereditary unconjugated hyperbilirubinemia, a disease first recognized by Crigler and Najjar (1952). CN-I is inherited as an autosomal recessive condition with an estimated frequency of less than one per million live births (Petit *et al.*, 2008). The clinical manifestation of CN-I is severe icterus. The serum bilirubin concentration associated with CN-I is greater than 20 mg/dl

(343 mmol/L), even 30–50 mg/dl (513–855 mmol/L), and the bilirubin is entirely unconjugated (Maruo *et al.*, 2011). Phenobarbital treatment is not effective for the treatment of symptoms. Patients with CN-I are in danger of kernicterus and must receive daily phototherapy immediately in the neonatal period. Liver transplantation is eventually required.

CN-I is caused by homozygous or compound heterozygous mutations of the UDP-glycosyltransferase gene (*UGT1A1*; MIM #191740) on chromosome 2q37 (Costa *et al.*, 2009), leading to a complete and non-inductible deficiency of bilirubin UDP-glucuronosyltransferase 1-1 (*UGT1A1*) activity. *UGT1A1* (ENSP00000304845) is a membrane

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enzyme of the endoplasmic reticulum comprising 533 amino acids, and is expressed only in the liver, esophagus, stomach, and intestine (Tukey and Strassburg, 2001; Basu *et al.*, 2004). The signal peptide of UGT1A1 is composed of amino acids 1–27; amino acids 29–468 constitute the superfamily domains; and, the transmembrane region is composed of amino acids 13–35 and 491–513 (Wilke *et al.*, 2000). UGT1A1 plays a critical role in the bilirubin-conjugation process (Tukey and Strassburg, 2001).

The first mutation described in *UGT1A1* in 1992 was a nonsense mutation found in a homozygous state in a patient with CN-I (Bosma *et al.*, 1995). To date, more than 130 mutations have been identified, most of which are missense or nonsense mutations (Canu *et al.*, 2013).

In this paper, we studied two CN-I patients from two unrelated families. Three mutations were identified: c.239_245delCTGTGCC (p.Pro80HisfsX6), c.1253delT (p.Met418ArgfsX5), and c.1156G>T (p.Val386Phe) of *UGT1A1*. Moreover, we confirmed that one patient had an entire deletion on one allele of the *UGT1A1* gene.

2 Materials and methods

2.1 Patient A

Patient A was 3.5 months old, a Chinese female of nonconsanguineous parents. She was the fifth fetus and the second newborn baby of her mother; the first three fetuses were voluntarily aborted, and the fourth fetus was her older deceased sister. Patient A was born at full term (weight 3600 g) by normal vaginal delivery following a normal pregnancy. At 3 d after birth, she began to demonstrate icterus; at 7 d, her serum total bilirubin level was 570.5 $\mu\text{mol/L}$ and unconjugated bilirubin level was 549.9 $\mu\text{mol/L}$, and she was admitted to the local hospital. She was treated with daily phototherapy, albumin infusion, and exchange blood transfusion. Upon treatment, her serum total bilirubin decreased to 169.2 $\mu\text{mol/L}$. Soon after the treatment, the bilirubin level increased. Yinzhihuang oral liquid was administered to decrease her bilirubin, but no effect was observed. She was subsequently admitted to the Affiliated Children's Hospital of Capital Institute of Pediatrics (Beijing, China). Upon admission, the physical examination revealed severe

icterus, a body weight of 9200 g, a head circumference of 42.5 cm, normal neuromotor development, and no signs of kernicterus. Laboratory findings were as follows: serum total bilirubin, 589.5 $\mu\text{mol/L}$ (3.4–20.0 $\mu\text{mol/L}$) (Olympus AU640, Japan); serum unconjugated bilirubin, 571.8 $\mu\text{mol/L}$; alanine aminotransferase, 41.2 U/L (0–40 U/L); aspartate aminotransferase, 34.8 U/L (0–40 U/L); hemoglobin, 114 g/L (110–190 g/L); and, reticulocyte count, 1.1% (0.5%–1.5%). Treatment with phenobarbital intramuscular injection for two days (4 mg/kg per day) did not reduce her serum bilirubin levels; therefore, the patient was discharged. The patient's outcome was unknown because of loss to follow-up. The serum bilirubin concentrations of the patient's parents were not measured, and they did not demonstrate jaundice; however, the patient's sister had severe hyperbilirubinemia after birth, experienced convulsions at 7 months, and died of kernicterus at 22 months.

2.2 Patient B

Patient B was 4.5 months old, a Chinese male of nonconsanguineous parents. He was the first child of the family. He was born at full term (weight 3200 g) by normal vaginal delivery, but his mother had been considering abortion during the pregnancy. Although his parents displayed no apparent jaundice, patient B appeared to be jaundiced at 6 d after birth and then developed severe icterus. At one month, his serum total bilirubin was 411.6 $\mu\text{mol/L}$ and unconjugated bilirubin was 403.1 $\mu\text{mol/L}$; he was admitted to the local hospital and treated daily with phototherapy. Upon treatment, the transcutaneous bilirubin decreased slightly (to 16.5 mg/dl). Bilirubin levels increased immediately when phototherapy stopped, and Yinzhihuang oral liquid was administered, but no significant reduction in bilirubin was observed. At four months of age, his serum total bilirubin was 520.6 $\mu\text{mol/L}$ and unconjugated bilirubin was 499.6 $\mu\text{mol/L}$, and he was transferred to the Affiliated Children's Hospital of Capital Institute of Pediatrics (Beijing, China) for further treatment. Upon admission, the physical examination revealed severe icterus, a body weight of 7000 g, a height of 66 cm, a head circumference of 41 cm, a slight retardation in mental and motor development (he could not raise his head), and no history of somnolence, vomit, convulsions, or opisthotonos. Laboratory findings were as follows:

serum total bilirubin, 496.5 $\mu\text{mol/L}$ (Olympus AU640, Japan); serum unconjugated bilirubin, 490.7 $\mu\text{mol/L}$; alanine aminotransferase, 34.1 U/L; aspartate aminotransferase, 21.8 U/L; and, hemoglobin, 111 g/L. Treatment with oral-phenobarbital for 8 d (10 mg/kg per day) did not reduce his serum bilirubin levels. At 13 months of age, patient B died of kernicterus.

2.3 DNA sequencing

Genomic DNA was isolated from the peripheral blood leukocytes of the patients and their parents, using the phenol-chloroform method (PC) (Sambrook *et al.*, 1989), with their informed consent. Five exons and their flanking sequences, specifically the promoter region of *UGT1A1*, were amplified with primers as described by Maruo *et al.* (1999). Then, direct sequencing of purified polymerase chain reaction (PCR) products was performed using an ABI 3700 automated sequencer (Applied Biosystems, USA).

2.4 Determination of *UGT1A1* gene copy number by quantitative real-time PCR (qRT-PCR)

Primers (Table 1) were designed using Primer Express 3.0 software (Applied Biosystems) for

qRT-PCR. P3–P8 were in *UGT1A1*. Primers of P1, P2, and P9 were designed to determine the deletion range of patient B. qRT-PCR was carried out using the ABI Prism™ 7500 sequence detection system and UltraSYBR Mixture (with ROX) (CW0956A, CWbio Co., Ltd., China) in reaction volumes of 25 μl . All reactions of the same run were prepared from the same master mix (2 \times UltraSYBR Mixture, 12.5 μl ; forward primer, 0.5 μl ; reverse primer, 0.5 μl ; DNA, 50 ng; add high performance liquid chromatography (HPLC)-pure water to 25 μl). Each pair of test and control samples was amplified in three different wells. Because there was no positive control, we conducted only a normal control. The PCR conditions were as follows: 10 min, 95 $^{\circ}\text{C}$; 40 cycles of 15 s at 95 $^{\circ}\text{C}$ and 1 min at 62 $^{\circ}\text{C}$; and, run for about 2 h (Wilke *et al.*, 2000).

Data evaluation was carried out using the ABI Prism™ 7500 sequence detection software. We used the $\Delta\Delta C_T$ method to analyze the gene copy number compared with that of healthy samples. The $2^{-\Delta\Delta C_T}$ was the ratio of inspected samples to controlled samples, specifically the ratio of target gene copy number to *GAPDH* copy number.

Table 1 Primers for qRT-PCR

Gene	Primer	Primer sequence (5'–3')	Product size (bp)
<i>USP40</i>	P1	F: GCCTTTGCCCTTCTTCCCTTT R: GAAGTAGCCCGCAACCAAAA	140
Upstream of <i>UGT1A8</i>	P2	F: AAGGCCTCTCAGGGTTTGGA R: GCCCTCAGCACTGACTTCCT	102
<i>UGT1A1</i>	P3 (PBREM)	F: CCGGACTTGGCACTTGGTAA R: GCCATTCTGGATCCCTTGCT	130
	P4 (Exon 1)	F: ACGTACCCTGTGCCATTCCA R: TGATCACACGCTGCAGGAAA	100
	P5 (Exon 2)	F: GCTGATGCTTTGGGCAAAA R: GGGATTAGCGCTCCTGTGAA	85
	P6 (Exon 3)	F: AGGTCCTGTGGCGGTACACT R: ATCCAATCCGCCAACATAC	110
	P7 (Exon 4)	F: CGATGACCCGTGCCTTTATC R: CGCTTTGCATTGTCCATCTG	118
<i>TRPM8</i>	P8 (Exon 5)	F: GCTATGAAGTGCTGGGCAAGT R: AAATGCGACACCAGTGAAAACA	110
		P9	F: CCAGGCTCAGCATGAGGAA R: TGCGCATGACTTACGCTTTC
<i>GAPDH</i>	Exon 4	F: AAGGCTGAGAACGGGAAGCT R: CCTAGTTGCCTCCCAAAGC	105

3 Results

3.1 Gene mutations in patient A and her parents

Mutations of the *UGT1A1* gene in patient A were c.239_245delCTGTGCC and c.1156G>T (Figs. 1a and 1b). The two mutations were observed in her parents, respectively, and therefore, were not spontaneous. Mutation c.239_245delCTGTGCC was predicted to cause an 85-amino acid truncated protein. To analyze the second mutation, c.1156G>T (p.Val386Phe), we first predicted this substitution base in ESEfinder Release 2.0 (<http://rulai.cshl.edu/tools/ESE2/>). We found that one default threshold value of wild sequence (CGTTCCCA) bound with SC35 protein was 2.529. However, this mutant sequence (1156G>T) (CTTTCCCA) was defective.

When the default threshold of SC35 protein is above 2.383, the 8-base sequence can bind with SC35 protein. This indicated that this missense mutation was meaningful and may affect RNA splicing by affecting binding with SC35. Then we compared the 30-amino acid residues 371–400 of UGT1A1 from ten species, and found that Val-386 of the human UGT1A1 was highly conserved and, therefore, likely to be an important amino acid. We also predicted mutation p.Val386Phe in SIFT (sorting intolerant from tolerant) and Polyphen software online, and the resulting scores were 0 (damaging) and 0.995 (deleterious). In addition, we searched c.1156G>T in the 1000 Genomes Project (<http://www.1000genomes.org/>), and found that this mutation was not a known polymorphic site.

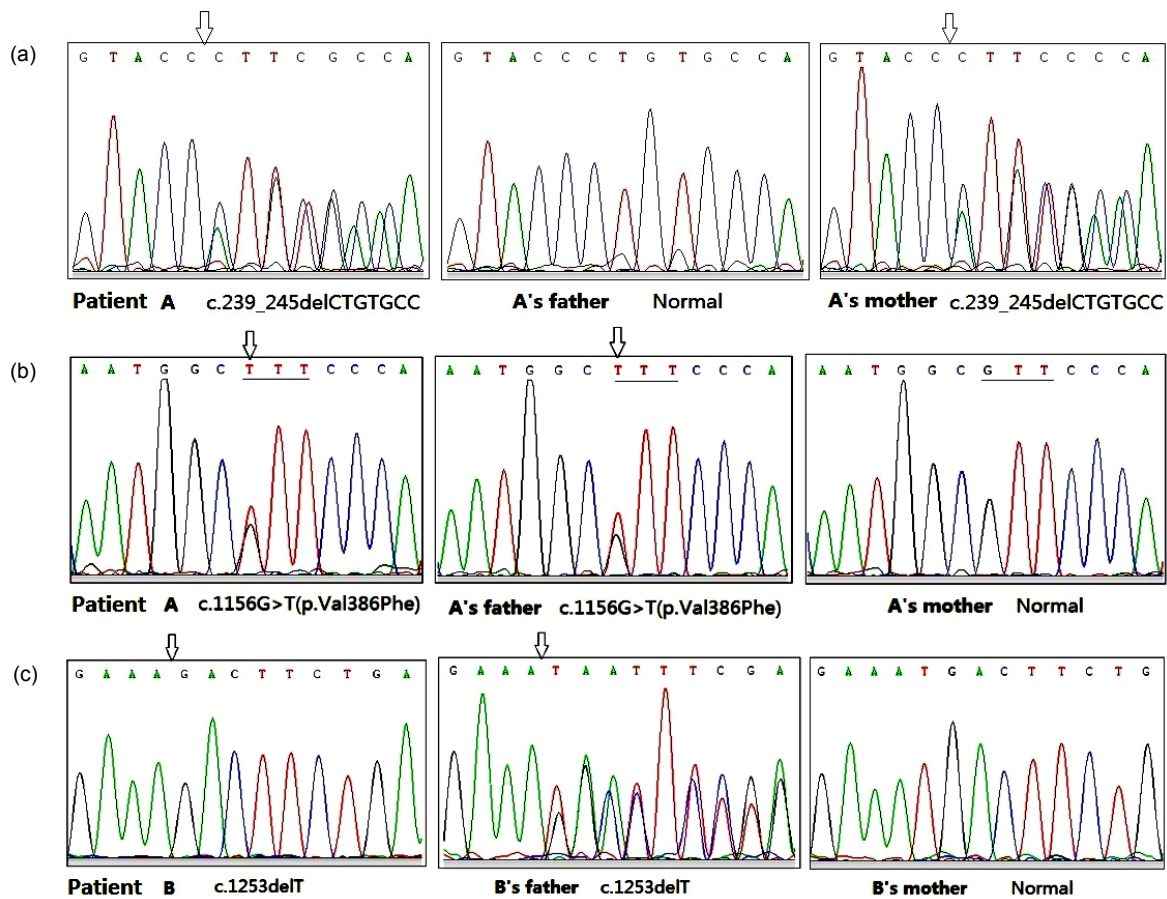


Fig. 1 Mutations of *UGT1A1* in the two patients

(a) In patient A: the deletion mutation c.239_245delCTGTGCC was found in patient A and her mother, and both were heterozygous; her father was normal at c.239_245. (b) In patient A: the missense mutation c.1156G>T (p.Val386Phe) was found in patient A and her father; her mother was normal at the same site. (c) In patient B: mutation c.1253delT in patient B was a homozygous deletion; his father had a heterozygous deletion; his mother was normal and had no base variation at the same site

3.2 Gene mutations in patient B and his parents

The mutation of the *UGT1A1* gene in patient B was c.1253delT, which looked from sequencing like a homozygous deletion. His father had a heterozygous deletion, but no base change was found at the same cDNA site in his mother (Fig. 1c). To explain the sequencing results, we postulated that there was a *UGT1A1* gene deletion in patient B's mother, and that this gene deletion was inherited by patient B. qRT-PCR was performed to verify our postulate (Fig. 2).

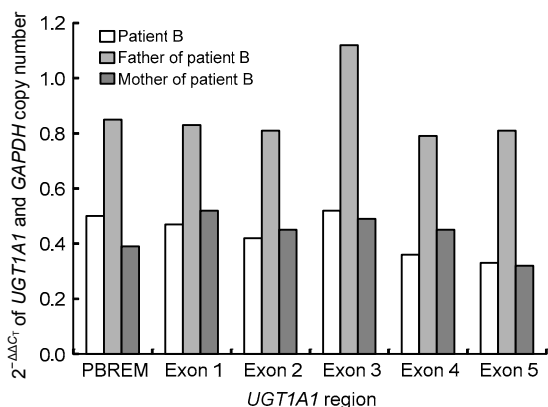


Fig. 2 qRT-PCR results for *UGT1A1* 2^{-ΔΔC_T} in family B. Patient B: 0.32–0.52; his mother: 0.32–0.52; his father: 0.79–1.12

The 2^{-ΔΔC_T} value of the normal control was considered to be 1. The father's values were between 0.79 and 1.12, similar to that of the control. The 2^{-ΔΔC_T} values of patient B and his mother were similar; their values were both between 0.32 and 0.52 from the upstream phenobarbital-responsive enhancer module (PBREM) region to exon 5, and were about half of the father's value. These data indicate that patient B and his mother had only one copy of the *UGT1A1* gene, i.e., they were hemizygous. Therefore, in patient B, one allele with an entire *UGT1A1* deletion was maternally inherited, and the other with mutation 1253delT was paternally inherited.

qRT-PCR results of three other regions (*USP40* and upstream of *UGT1A8* and *TRPM8*) showed that the *USP40* and upstream region of *UGT1A8* had two copies (1.00 and 1.01) and *TRPM8* had only one copy (0.47). Thus, we could speculate that the deletion extended at least 169.7 kb from the PBREM region of *UGT1A1* to *TRPM8* (Fig. 3).

Searches of the databases of National Center for Biotechnology Information (NCBI), Ensembl, and Human Gene Mutation Database (HGMD) up to October 2013 showed no reports of mutations c.239_245delCTGTGCC and c.1253delT. The main clinical manifestations and molecular analyses of the two CN-I patients are summarized in Tables 2 and 3, respectively.

4 Discussion

For patient A, RNA from mutation c.239_245delCTGTGCC was probably degraded by an RNA decay phenomenon, and a functional protein was also probably deficient.

On the other hand, a truncated protein of 85 amino acids (p.Pro80HisfsX6) was translated by RNA from this mutation, but lost most of the functional domain (p.86–p.533) of the *UGT1A1* enzyme. Clearly, this allele of *UGT1A1* with mutation c.239_245delCTGTGCC had lost gene function. The other allelic mutation, Phe-386 (c.1156G>T), substitutes the highly conserved Val-386 of *UGT1A1*. To predict its pathogenic mechanism, we conducted predictions of RNA splicing and protein function. First, we found that this missense mutation might affect RNA splicing by affecting binding with SC35 protein. However, it has been reported that *UGT1A1* is expressed only in the liver, esophagus, stomach, and intestine (Tukey and Strassburg, 2001; Basu *et al.*, 2004). So we could not verify this effect by trial. Predictions at the protein level indicated that most amino acids from 370 to 400 were highly conserved. Previous studies indicated that p.Ser376Phe (c.1124C>T), p.Ser381Arg (c.1143C>G), and p.Pro387Ala or p.Pro387Leu (c.1159C>G, c.1160C>T) mutations resulted in an inactive *UGT1A1* enzyme (Kadacol *et al.*, 2000; Sneitz *et al.*, 2010). This showed that the region from 370 to 400 amino acids is important.

On the other hand, the SIFT (0) and Polyphen (0.995) scores showed that the p.Val386Phe missense mutation was deleterious. However, a known missense mutation at this site, p.Val386Ile, found in Gilbert syndrome (Canu *et al.*, 2013), with a SIFT score of 0.210 and a Polyphen score of 0.665, does not lead to deleterious symptoms. According to clinical results and software prediction data, mutation p.Val386Phe is more deleterious than p.Val386Ile.

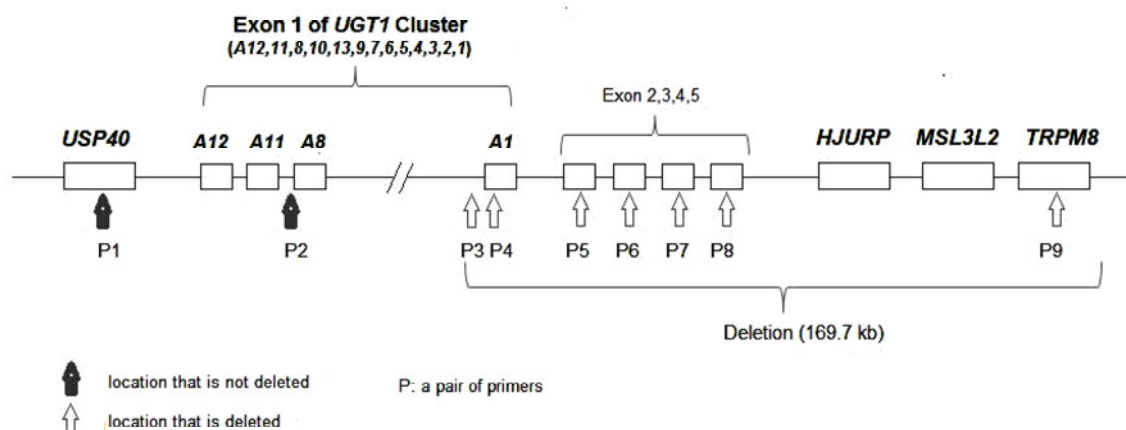


Fig. 3 Locations of primers and a draft genomic deletion map encompassing the *UGT1A1* gene and flanking regions in patient B

The maternal chromosome 2 from P3 to P9 is deleted; P1 and P2 are not deleted. The length of the deletion from the PBREM region of *UGT1A1* to *TRPM8* is 169.7 kb

Table 2 Main clinical manifestations of the two CN-I patients

Patient	Gender	Age (month)	Kernicterus	Serum bilirubin ($\mu\text{mol/L}$)		Phenobarbital treatment	Outcome
				Total	Unconjugated		
A	Female	3.5	No	589.5	571.8	Not effective	Unknown
B	Male	4.5	Uncertain	496.5	490.7	Not effective	Died of kernicterus at 13 months

Table 3 Molecular analyses of the two CN-I patients

Patient	<i>UGT1A1</i> gene	cDNA change	Amino acid change	Location	Mutation type	Source	(TA) polymorphism
A	Allele 1	c.1156G>T	p.Val386Phe	Exon 4	Missense	Paternal	(TA) ₆
	Allele 2	c.237_243del CTGTGCC	p.Pro80HisfsX6*	Exon 1	Frame shift	Maternal	(TA) ₇
B	Allele 1	c.1253delT*	p.Met418ArgfsX5*	Exon 4	Frame shift	Paternal	(TA) ₆
	Allele 2	Deletion of whole gene				Maternal	

* Novel mutation of *UGT1A1*

Valine and isoleucine are aliphatic, neutral, hydrophobic amino acids, while phenylalanine is an aromatic, neutral, hydrophobic amino acid. While p.Val386Ile likely affects the enzyme activity, perhaps it can be tolerated, because isoleucine and valine have similar structures. Although the properties of valine and phenylalanine are similar, steric hindrance from the large phenyl side chain of Phe, may affect the secondary structures of the protein. Petit *et al.* (2004) identified a CN-I patient with homozygous p.Val386Phe, and also indicated that p.Val386Phe was damaging. In conclusion, we believe that the missense mutation p.Val386Phe is likely to result in an inactive enzyme on account of the manifestation of

severe icterus, non-effective phenobarbital treatment, prediction results, and a truncated inactive enzyme formed by the other allele. However, the pathogenic mechanism of the p.Val386Phe mutation remains to be determined.

For patient B, the maternal *UGT1A1* allele was deleted entirely. A 422-amino acid truncated protein (p.Met418ArgfsX5) was predicted as a result of the c.1253delT mutation of the paternal allele, lacking 111 amino acids of the *UGT1A1* C-terminus, and predicted to lack the transmembrane domain. Also, abnormal RNA produced by mutation c.1253delT was probably degraded by an RNA decay pathway. Because patient B had severe icterus, phenobarbital

treatment was not effective, and he died of kernicterus at 13 months, we concluded that the paternal allele with the c.1253delT mutation had lost its function. Because sequencing results did not completely explain the base variation at c.1253delT found in family B, we explored and confirmed the possibility of an allelic deletion of the *UGT1A1* gene. The length of the deletion of patient B's maternal chromosome 2 was speculated to be at least 169.7 kb. To our knowledge, entire allelic deletions identified in CN-I patients have rarely been reported.

The two CN-I patients in this study were treated with daily phototherapy, albumin infusion, and exchange transfusion. These therapies temporarily decreased bilirubin levels. As patients were hospitalized for only several days, some more effective therapies, such as liver transplantation and hepatocyte transplantation (Lucey et al., 2000; Lysy et al., 2008), were not applied. In the near future, gene therapy may be a prospective therapeutic method (Bortolussi et al., 2012).

In summary, we identified three mutations, c.239_245delCTGTGCC (p.Pro80HisfsX6), c.1253delT (p.Met418ArgfsX5), and c.1156G>T (p.Val386Phe), and an entire *UGT1A1* gene deletion, in two CN-I patients. The former two mutations were novel and were considered as pathogenic mutations. While software predictions showed that the third mutation, c.1156G>T, affects RNA splicing and protein function, its pathogenic mechanism remains unknown. We also confirmed one patient had lost heterozygosity of the *UGT1A1* gene by inheriting a deletion of one entire allele.

Acknowledgements

We would like to thank the two patients and their family members for their cooperation in this study.

Compliance with ethics guidelines

Yan LI, Yu-jin QU, Xue-mei ZHONG, Yan-yan CAO, Li-min JIN, Jin-li BAI, Xin MA, Yu-wei JIN, Hong WANG, Yan-ling ZHANG, and Fang SONG declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all

patients for which identifying information is included in this article.

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中文概要:

本文题目: 两例无关的罕见 I 型 Crigler-Najjar 综合征患者: 两种新突变和一例患者 *UGT1A1* 基因杂合性缺失

Two unrelated patients with rare Crigler-Najjar syndrome type I: two novel mutations and a patient with loss of heterozygosity of *UGT1A1* gene

研究目的: I 型 Crigler-Najjar 综合征 (CN-I) 为先天性间接胆红素血症的最严重的一种, 是由位于染色体 2q37 的葡萄糖醛酸转移酶基因 (*UGT1A1*) 的纯合或复合杂合突变引起的一种罕见的遗传性疾病。本研究对来自两个无关家庭的两例临床诊断为 CN-I 的患儿及父母进行 *UGT1A1* 基因分子遗传学分析。

研究方法: 经知情同意后, 采集两例患儿及父母外周血; 聚合酶链式反应 (PCR) 扩增 *UGT1A1* 基因 5 个外显子及外显子-内含子交界处, 进行测序分析。应用实时定量 PCR (qRT-PCR) 测定其中一例患者 *UGT1A1* 基因的拷贝数。

重要结论: 本研究在两例 CN-I 型患儿中检测到 3 个 *UGT1A1* 基因突变: c.239_245delCTGTGCC (p.Pro80HisfsX6)、c.1253delT (p.Met418ArgfsX5) 和 c.1156G>T (p.Val386Phe)。前两个突变均为新发的移码突变, 预测提前出现终止密码或诱发 RNA 降解; 而突变 c.1156G>T (p.Val386Phe) 的致病机制尚需进一步研究。

关键词组: I 型 Crigler-Najjar 综合征 (CN-I); 高胆红素血症; UDP-葡萄糖醛酸转移酶基因 (*UGT1A1*); 突变; 杂合性缺失