Molecular analysis of patients of Sardinian descent with Crigler-Najjar syndrome type I

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Abstract

This study reports the molecular characterisation of the bilirubin UDP-glucuronosyl-transferase gene (UGT1) in a group of patients of Sardinian descent with Crigler-Najjar syndrome type I and their relatives.

Sequence analysis of both UGT1A exon 1 and common exons 2-5 was performed in all patients, leading to the detection of Δ F170 and a novel mutation (470insT), both residing in UGT1A exon 1. All but two heterozygotes for the Δ F170 mutation showed normal serum bilirubin levels. These two subjects were also heterozygous for the sequence variation A(TA)₇TAA in the promoter region of the UGT1A gene.

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Crigler-Najjar (CN) syndrome is an inborn error of metabolism, which is characterised by severe unconjugated hyperbilirubinaemia¹ resulting from defective activity of the hepatic enzyme bilirubin UDP-glucuronosyl-transferase (UGT). CN syndrome has been classified into two types according to the degree of hyperbilirubinaemia and response to phenobarbital administration.² In type I, the level of unconjugated bilirubin in serum is usually above 340 μ mol/l and phenobarbital treatment does not reduce it significantly. Type II patients have serum bilirubin levels in the 120-340 μ mol/l range and respond to phenobarbital treatment by a decrease of at least 25% of bilirubin levels. CN-I is inherited as an autosomal recessive disorder, whereas for type II both autosomal recessive and dominant inheritance with variable penetrance have been reported.

The recent identification of a large gene locus termed UGT1, which encodes a family of UDP-glucuronosyl-transferase (UGT1A-UGT1M) including the two bilirubin transferase isoforms, has provided the tools for studying hereditary/unconjugated hyperbilirubinaemia at the molecular level.^{3,4} The complex UGT1A-UGT1M codes for at least two bilirubin transferase, three bilirubin-like, and eight phenol-transferase isozymes.⁵ In the 5' region of the locus, a minimum of 13 different exons 1 are located, each with an upstream specific promoter, while the 3' region contains four common exons. Each exon 1 encodes the amino-terminus of a specific transferase and the common exons (2-5) encode the common

carboxyl-terminus of each isoform. The product of the UGT1A gene is the predominant bilirubin isoform in liver, while the product of the UGT1D gene is the less abundant and phenobarbital responsive bilirubin transferase isoenzyme.

Mutation analysis in patients with Crigler-Najjar type I has detected deletions, nonsense and missense mutations most commonly in the common exons and occasionally in the unique specific exon of the UGT1A unit. This finding of mutations in the specific exon 1, together with the transfection experiments in COS cells, indicates that the UGT1A unit codes for the only relevant isoform in bilirubin glucuronidation.⁶ In the few patients with CN-II analysed molecularly to date, missense mutations in the specific exon 1 of the UGT1A unit, or in common exons, have been detected, in some cases associated with missense mutations of unique exon 1 of the UGT1D gene.⁷⁻⁹

More recently, patients with Gilbert syndrome, the mildest of the inherited hyperbilirubinaemias, were found to be either heterozygous for missense mutations in the specific exon 1 of UGT1A or in the common exon 4 or 5,^{10,11} or homozygous for an extra TA dinucleotide in the TATA sequence of the promoter region of UGT1A (A(TA)₇TAA instead of the normal A(TA)₆TAA).¹²⁻¹⁴

Here, we describe the results of the molecular characterisation of the UDP-glucuronosyl-transferase gene in a group of patients of Sardinian descent with Crigler-Najjar syndrome type I and their relatives.

Patients

We have examined five patients with severe unconjugated hyperbilirubinaemia belonging to four families of Sardinian descent. All patients, who were born to non-consanguineous parents, had persistent non-haemolytic hyperbilirubinaemia (level >250 μ mol/l). One patient (PE) required an exchange transfusion during the neonatal period because of a sudden increase of bilirubin levels (547 μ mol/l). An exchange transfusion was carried out in infancy in patient PL, whose bilirubin levels increased up to 684 μ mol/l with associated signs of neurological dysfunction (slurring of speech, drooling, reduced control of truncal tone, abnormality of fine hand movements, and moderate ataxia). This patient also had a mild form of dyskinetic central palsy with intelligence in the low-normal range. The bilirubin levels in all patients were unresponsive to phenobarbital administration. Limited

Table 1 Relevant clinical features of Crigler-Najjar type I patients

Patients	Age (y)	Genotype	Max bilirubin levels		Exchange transfusions		Neurological dysfunction	Liver transplantation
			Neonatal period	Infancy/childhood	Neonatal period	Infancy/childhood		
CR	13	ΔF170/ΔF170	273		None	None	Absent	Yes
PL	8	ΔF170/ΔF170	342	684	None	One	Present	Yes
PE*	16	ΔF170/470insT	547		One	None	Present	No
PA*	4	ΔF170/470insT	256	291	None	None	Absent	No
PEd	1	ΔF170/ΔF170	308	—	None	—	Absent	No

*Sibs.

Figure 1 Family pedigree of PED. II.6 and II.7 have Gilbert syndrome. III.10 is the proband homozygous for the ΔF170 mutation. 6/6 genotype = A(TA)₆TAA/A(TA)₆TAA. 6/7 genotype = A(TA)₆TAA/A(TA)₆TAA. * = STB concentration (μmol/l). * above families = members not tested.

reduction of the serum bilirubin levels was observed with phototherapy, which was used continuously in all the patients. Patient PL also required sporadic plasmapheresis to reduce the toxic high bilirubin levels. Liver biopsy was carried out in patient PL, in whom hepatic UDP glucuronosyl-transferase was absent. In two patients (CR and PL), aged 12 and 6½ years respectively, orthotopic liver transplantation was carried out. After transplantation, the patients' serum bilirubin levels rapidly returned to normal. These data are summarised in table 1. In six relatives of one of the patients investigated (PEd), who were found to be heterozygous for a mutation in the UDP glucuronosyl-transferase gene, we carried out serum total bilirubin determination (STB). The results were normal in all but two, who had been previously diagnosed as affected by Gilbert syndrome (fig 1).

Mutation analysis

Sequence analysis of both the specific exon 1 of UGT1A and the common exons 2-5 in all the five patients showed two mutations in exon 1, the previously described deletion of the phenylalanine codon (TTC) at position 170 (ΔF170) of UGT1A and a novel mutation, the insertion of a T at codon 158-159 (470insT), which results in a frameshift and in the production of a downstream stop codon (TGA) at position 182 (fig 2). Of these patients, three unrelated ones were homozygotes for ΔF170 and two sibs were compound heterozygotes for ΔF170 and 470insT. Of the 10 chromosomes carrying a defective UGT1A gene, eight thus contained the ΔF170 mutation.

The ΔF170 mutation may be easily detected by electrophoretic separation of a 401 bp DNA fragment containing the mutation on a 2 mol/l urea, 6% polyacrylamide gel. The 401 bp fragment was amplified with forward primer

Figure 2 Sequence analysis of UGT1A exon 1 gene for the identification of the 470insT mutation. The arrow indicates the localisation of the mutation. N and M show the normal and mutant sequences respectively.

AGCTCATGGCCTCCCT and reverse primer GATGGGCCTAGGGTAAT for 25 PCR cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for one minute in a Perkin-Elmer Cetus Thermal Cycler 9600 (fig 3). This procedure was used for screening the relatives of patient PED. Six of them, hetero-

Figure 3 Polyacrylamide gel electrophoresis of a 401 bp PCR product containing part of UGT1A exon 1. Lane 1 normal subject, lane 2 proband homozygous for $\Delta F170$ mutation, lanes 3 and 4 parents heterozygous for the $\Delta F170$ mutation.

zygous for the $\Delta F170$ mutation, were investigated for the A(TA)₇TAA nt variation. Only the two subjects with increased STB concentration in this group showed heterozygosity for A(TA)₇TAA, the others being homozygous for A(TA)₆TAA.

Discussion

In this study, we have characterised the molecular defect in the bilirubin UDP-glucuronosyl transferase gene in five patients affected by Crigler-Najjar syndrome type I belonging to four families of Sardinian origin. We found three patients homozygous for a previously described mutation, the deletion of the phenylalanine codon at position 170 ($\Delta F170$) of exon 1 of UGT1A, and two patients from the same family compound heterozygous for $\Delta F170$ and a novel mutation consisting of the insertion of a T at codon 158-159 (470insT) of exon 1 of UGT1A.

The $\Delta F170$ mutation has been previously described in a single patient of caucasian origin.¹⁵ This mutation abolishes a conserved diphenylalanine at this position and results in the production of one third level of mRNA and presumably mutant protein as compared to the wild type gene. Furthermore, the mutant protein has no enzymatic activity at low pH (6.4), which is the optimum pH for bilirubin glucuronidation.

The 470insT mutation results in a frameshift and downstream stop codon, thus probably leading to absent protein product or to the production of a shortened functionless protein. Both mutations detected in this study reside in

the specific exon 1 of UGT1A. This finding is consistent with the notion that UGT1A codes for the only relevant enzymatic isoform in bilirubin glucuronidation.

It is interesting to note that seven of eight mutant alleles from unrelated patients of Sardinian ancestry carry the $\Delta F170$ mutation. This may indicate that Crigler-Najjar syndrome type I in Sardinia is prevalent because of a founder effect. A similar effect has been previously detected for Crigler-Najjar syndrome type I in France, Portugal, Turkey, and Tunisia.¹⁶

In six heterozygotes, all belonging to the same family, STB levels and promoter sequence variation were studied. Four of these have normal STB levels and absence of the promoter variation A(TA)₇TAA. Normal STB concentration has to date been reported in subjects heterozygous for mutations that, most likely, cause absence of protein product or production of a shortened functionless protein. Tetramers are thus assembled only from the normal subunits, probably resulting in 50% normal activity, which may be sufficient for bilirubin metabolism in normal conditions. This could be a reason for the normal STB values shown by these subjects. Two of the $\Delta F170$ heterozygotes examined showed high STB levels (>40 $\mu\text{mol/l}$) and the presence of the sequence variation A(TA)₇TAA in the heterozygous state. The sequence variation, although found in the heterozygous state, could explain the mild increase of STB concentration in these subjects. Recently it has been reported that a consistent decrease of the enzymatic activity of the UGT1A gene may also occur in those patients with Gilbert syndrome who are homozygous for the variant A(TA)₇TAA promoter.¹² The transfection of a construct containing the A(TA)₇TAA promoter linked upstream to the firefly luciferase gene in a hepatic cell line led to a reduced luciferase expression, that is, 18 to 32% of that recorded in the presence of the normal TATA sequence motif (A(TA)₆TAA).

Further molecular analysis and mutation-phenotype correlation of patients with CN-I, CN-II, and Gilbert syndrome are needed to have a clear picture of the molecular pathogenesis of these inherited hyperbilirubinaemias.

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