Case Report

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Disruption of HNF1α binding site causes inherited severe unconjugated hyperbilirubinemia

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Abstract

Crigler-Najjar syndrome presents as severe unconjugated hyperbilirubinemia and is characteristically caused by a mutation in the *UGT1A1* gene, encoding the enzyme responsible for bilirubin glucuronidation. Here we present a patient with Crigler-Najjar syndrome with a completely normal *UGT1A1* coding region. Instead, a homozygous 3 nucleotide insertion in the *UGT1A1* promoter was identified that interrupts the HNF1α binding site. This mutation results in almost complete abolishment of *UGT1A1* promoter activity and prevents the induction of UGT1A1 expression by the liver nuclear receptors CAR and PXR, explaining the lack of a phenobarbital response in this patient. Although animal studies have revealed the importance of HNF1α for normal liver function, this case provides the first clinical proof that mutations in its binding site indeed result in severe liver pathology stressing the importance of promoter sequence analysis.

Introduction

A 25 year old female patient from Turkey presented with serum unconjugated bilirubin (UCB) levels around 300 μmol/L and conjugated bilirubin around 15 μmol/L, in line with Crigler-Najjar syndrome (CNS) type II [1]. In contrast to patients with CNS type II, this patient had not responded to administration of phenobarbital (50 mg/kg) in the past, an established treatment for CNS type II patients. Therefore, the patient had been treated with phototherapy (4 h/day), which reduced serum bilirubin levels to about 200 μmol/L.

The severe unconjugated hyperbilirubinemia characteristic for CNS is caused by deficiency of hepatic UGT1A1 activity [2]. In contrast to type I, patients with CNS type II do have some residual UGT1A1 activity that can be induced by phenobarbital administration via activation of the constitutive androstane receptor (CAR; NR1I3) resulting in a >30% reduction of serum bilirubin levels. This transcriptional activation of the *UGT1A1* promoter by CAR depends on its binding to the specific binding site in the gtPBREM region located between −3499/−3210 and the HNF1α binding site between −75 and −95 present in the *UGT1A1* promoter. To clarify the cause of UGT1A1 deficiency and the non-responsiveness to phenobarbital a detailed genetic analysis was performed.

Methods

UGT1A1 coding and promoter sequence analysis

Genomic DNA was isolated from the patient and her parents. The entire coding region, the splice sites, the proximal and the distal gtPBREM promoter regions of the *UGT1A1* gene were amplified using Taq-polymerase (Promega, Madison, USA). For primer sequence see Supplementary Table 1. Amplicons were isolated from 1% agarose (Seakem LE agarose, Rockland, USA) electrophoresis gel by Zymoclean Gel DNA recovery kit (Zymo Research, Irvine, USA) and sequenced using BigDye Terminator v1.1 (Life technologies, Carlsbad, USA).

Cell culture

The human hepatoma cell line HepG2, overexpressing CAR (HepG2^{CAR}) was generated by transduction with a lentiviral vector encoding the CAR cDNA behind the constitutive CMV promoter, HepG2 overexpressing pregnane X receptor (PXR) (HepG2^{PXR}) behind a PGK promoter was generated previously [3]. HepG2, HepG2^{CAR}, HepG2^{PXR}, and HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Lonza, Cologne, Germany) supplemented with 10% foetal calf serum (FCS), 4 mmol/L L-glutamine and a mixture of antibiotics (5 mg/ml penicillin, 5 mg/ml streptomycin).

Keywords: Promoter mutation; Crigler-Najjar syndrome; UGT1A1; Transcriptional regulation; HNF1α.
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Abbreviations: CAR, Constitutive androstane receptor; CNS, Crigler-Najjar syndrome; gtPBREM, phenobarbital-responsive enhancer module; HNF1α, Hepatic nuclear factor 1α; NT, nucleotide; PXR, Pregnane X Receptor; UCB, unconjugated bilirubin; UGT1A1, Uridine Diphosphate Glucuronosyltransferase 1A1; FCS, foetal calf serum.

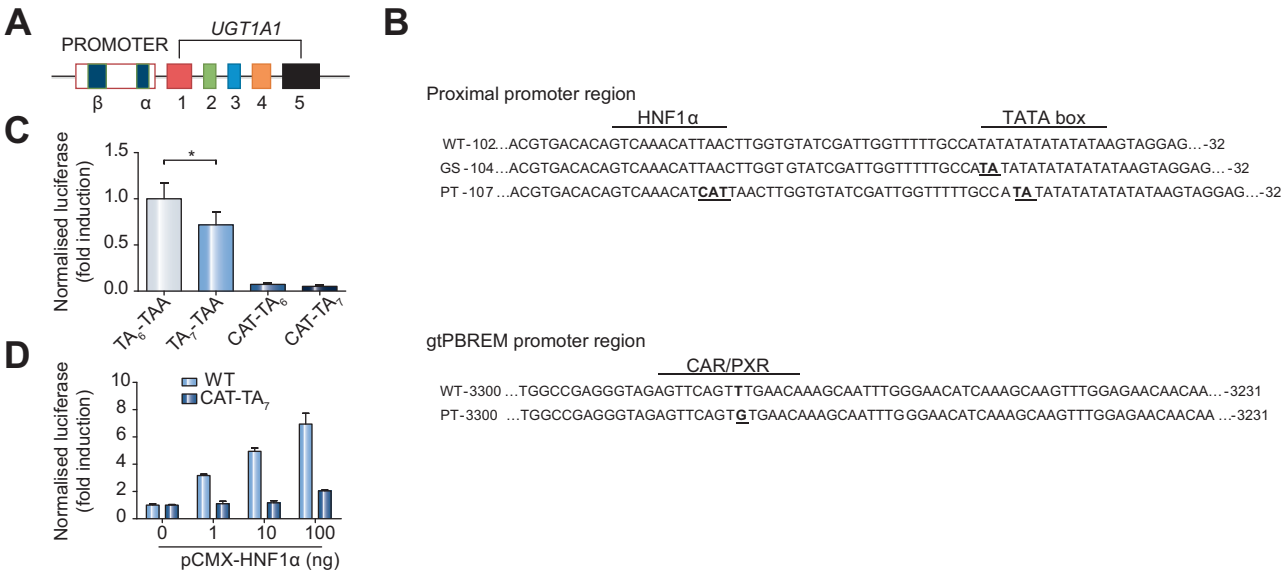

Fig. 1. *UGT1A1* gene structure and sequence and the effects of SNP on UGT1A1 activity. (A) Human *UGT1A1* gene structure. α proximal promoter region (−181/−1) containing the HNF1α binding site and the TATA box. β distal gtPBREM module (−3499/−3210). (B) *UGT1A1* gene promoter sequence. Sequences of the proximal promoter of a control (wt) of a Gilbert syndrome (GS) and of the patient (PT) indicating the HNF1α-binding site and the TATA box, and the distal gtBREM promoter indicating the −3279T>G SNP are shown. (C) Activity of the UGT1A1 proximal promoter in HepG2 cells. HepG2 cells were transfected with luciferase reporter plasmids containing *UGT1A1* gene proximal promoters with the wt (TA_6–TAA), the Gilbert (TA_7–TAA), the CAT insertion in the HNF1α binding site (CAT-TA_6) and the patients sequence (CAT insertion and TA_7TAA (CAT-TA_7)) and a plasmid expressing renilla luciferase. Data represent the mean ± SD of the firefly-renilla luciferase ratio (RLX/RLU) determined 48 h after transfection, obtained in four independent experiments (n = 4) $^{*}p$ <0.05, One-way analysis of variance (ANOVA) was used for statistical analysis. (D) HNF1α does not activate mutated *UGT1A1* gene proximal promoter. HEK293T were transfected with luciferase reporter plasmids containing the wt (light blue bar) or the mutated CAT-TA_7 (dark blue bar) *UGT1A1*-proximal promoter, increasing amounts of CMV-HNF1α or an empty control plasmid and a renilla luciferase plasmid. Data represent the mean ± SD of the firefly-renilla luciferase ratio (RLX/RLU) determined 48 h after transfection, obtained in two independent experiments (n = 2). (This figure appears in colour on the web.)

Functional analysis of UGT1A1 promoter activity by luciferase reporter assay

UGT1A1 proximal promoter fragments with the wild-type (wt) allele (6 TA repeats) or the *UGT1A1**28 allele with 7 TA repeats with and without the CAT insertion were amplified by PCR from genomic DNA using Phusion high-fidelity Polymerase (New England Biolabs Inc) and cloned into a pGL4.22 [luc2CP/PURO] vector (Promega). The gtPBREM region was amplified from genomic DNA and inserted upstream of the various proximal constructs. All constructs were sequenced to confirm absence of mutations (for primer sequence see Supplementary Table 1).

Transient transfection was used to determine transcriptional activity of the different promoter constructs. HepG2, HepG2^{CAR}, HepG2^{PXR}, and HEK293T cells were plated at a confluence between 40 to 60%, and 24 h later transfected with 1 μg of a luciferase reporter plasmid and 25 ng of a renilla plasmid (to allow normalization of transfection efficiency), using polyethyleneImine (PEI) in DMEM without FCS. Four hours later the transfection medium was replaced with DMEM supplemented with 10% FCS. Promoter activity was measured 48 h after transfection using the Dual-Luciferase Reporter Assay system (Promega). To show the effect of HNF1α, co-transfection with an HNF1α expressing plasmid (a kind gift of Prof. Dr P. Mackenzie) was performed.

Induction of endogenous UGT1A1 and UGT1A1 promoter constructs by phenobarbital and rifampicin

HepG2 and HepG2 transfected with the *UGT1A1* promoter constructs were cultured overnight in serum-free medium containing 0.2% BSA. The next day the cells were washed and incubated at 37 °C in 5% CO_2 with DMEM/0.2% BSA containing rifampicin (10 μm/L) or phenobarbital (2 mmol/L) or 0.1% dimethyl sulfoxide (DMSO) as a solvent control, 24 h thereafter cells were harvested. RNA extraction, cDNA synthesis and quantitative PCR were performed as described previously [8]. Induction of the promoter activity was measured using the Dual-Luciferase Reporter Assay system (Promega). Rifampicin, phenobarbital and DMSO were purchased from Sigma Aldrich (Steinheim, Germany).

Results

Genetic analysis of the UGT1A1 gene and promoter

No mutations were found in the coding region and the splice donor and acceptor sites of exons 1 to 5, indicating that the gene encodes the normal UGT1A1 protein. In the *UGT1A1* promoter region of the patients several sequence abnormalities were identified (Fig. 1B). The patient appeared to be homozygous for the UGT1A1*28 allele, an extra TA in the TATAA box, corresponding to the genotype present in Caucasians with Gilbert syndrome [4]. In addition, the patient was found to be homozygous for a common single nucleotide polymorphism (SNP) −3279T>G in the gtPBREM site. The combination of these two sequence abnormalities has been reported to decrease the transcription of the *UGT1A1* gene by 50% [5]. This reduction however is not severe enough to cause the high serum UCB concentration seen in this patient.

In addition to these two common sequence abnormalities the patient appeared homozygous for a 3 nucleotide (nt) (CAT) insertion between position −83 to −85 of the proximal promoter region (Fig. 1B). This insertion interrupts the HNF1α binding site that is located between −79 to −95 in the *UGT1A1*-promoter [6]. Both parents appeared heterozygous carriers of the UGT1A1*28 allele and the 3nt CAT insertion, indicating both mutations are present in the same allele. To distinguish the role of all three *UGT1A1* promoter sequence abnormalities in the phenobarbital-unresponsive severe hyperbilirubinemia seen in this patient, functional studies were performed.