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Title Page

*****Short Communication*****

(Submission to journal: Drug Metabolism and Disposition)

A Novel Pathogenic *UGT1A1* Variant in a Sudanese Child with Type I Crigler-Najjar Syndrome

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Running Title page

a) Running Title: A Novel pathogenic UGT1A1 variant

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h) List of abbreviations:

UGT: Uridine diphosphate glucuronosyltransferase

CN-I: Crigler Najjar Type I

WT: wild type

Abstract

Uridine diphosphate glucuronosyltransferases (UGTs) are key enzymes responsible for the body's ability to process of a variety of endogenous and exogenous compounds. Significant gains in the understanding of UGT function have come from the analysis of variants seen in patients. We cared for a Sudanese child who presented with clinical features of Type I Crigler-Najjar syndrome (CN-I), namely severe unconjugated hyperbilirubinemia leading to liver transplantation. CN-I is an autosomal recessive disorder caused by damaging mutations in the gene for UGT1A1, the hepatic enzyme responsible for bilirubin conjugation in humans. Clinical genetic testing was unable to identify a known pathogenic *UGT1A1* mutation in this child. Instead, a novel homozygous variant resulting in an in-frame deletion, Val275del, was noted. Sanger sequencing demonstrated that this variant segregated with the disease phenotype in this family. We further performed functional testing using recombinantly expressed UGT1A1 with and without the patient variant, demonstrating that Val275del results in a complete lack of glucuronidation activity, a hallmark of CN-I. Sequence analysis of this region shows a high degree of conservation across all known catalytically active human UGTs, further suggesting that it plays a key role in the enzymatic function of UGTs. Finally, we note that the patient's ethnicity likely played a role in his variant being previously undescribed, and advocate for greater diversity and inclusion in genomic medicine.

Introduction

Uridine diphosphate glucuronosyltransferases (UGTs) comprise a superfamily of enzymes that conjugate a variety of endogenous and exogenous molecules with the glucuronyl group from the donor UDP-glucuronic acid. Mostly, this conjugation reaction reduces lipophilicity and results in excretion of these compounds from the human body (Hu et al., 2016). It is increasingly recognized that polymorphisms in UGTs can not only affect the metabolism rate of a variety of medications, but could also predispose their carrier to a variety of malignancies, possibly by altering the processing of potential carcinogens (Hu et al., 2016; Sanchez-Dominguez et al., 2018). Understanding the enzymatic function of UGTs, therefore, is of key importance to a variety of fields including pharmacogenomics, drug development, cancer risk assessment and cancer therapeutics.

Among known UGT enzymes, the UGT1 and UGT2 families are the most biologically relevant. UGT1A1 in particular has been well studied due to its unique role as the sole enzyme in humans responsible for the conversion of bilirubin into its more water-soluble mono- and di-glucuronidated forms, both of which are readily excreted into bile (Erlinger et al., 2014; Memon et al., 2016). Although the enzyme is involved in the metabolism of various other endogenous and exogenous substrates, the study of *UGT1A1* mutations in patients with hyperbilirubinemia has greatly contributed to our understanding of the enzymatic function of UGTs.

Mutations in the coding region of *UGT1A1* can lead to Crigler-Najjar syndrome (CN), whereas mutations in the promoter region can result in reduced enzyme expression and the milder unconjugated hyperbilirubinemia seen in Gilbert syndrome. Type I CN (CN-I), with complete absence of hepatic UGT1A1 activity, is potentially fatal. These children present in the

first few days of life with severe jaundice and extremely elevated bilirubin of greater than 20 mg/dL (normal <1 mg/dL), placing them at high risk for kernicterus and neurologic impairment. They are managed with intensive phototherapy, typically 12 hours daily, which converts trans-bilirubin to its more water-soluble cis-bilirubin isomer. This therapy has significantly improved survival, though skin thickening due to chronic phototherapy typically occurs in the teenage years, rendering phototherapy ineffective. Currently, the only definitive treatment for CN-I is liver transplantation (Fagiuoli et al., 2013). Type II CN (CN-II) is characterized by a partial reduction in enzyme activity, typically resulting in a later presentation with jaundice and bilirubin levels of <20 mg/dL. Patients with CN-II can be managed with phenobarbital, which is able to increase the effective activity of their defective enzyme by boosting total UGT1A1 expression, whereas CN-I patients, having completely absent enzyme activity, have no response to phenobarbital.

To date, mutations affecting 34 distinct amino acid residues of UGT1A1, either in the homozygous or compound heterozygous state, have been associated with CN-I, as noted from the Human Gene Mutation Database (Stenson et al., 2014). Here we describe the case of a Sudanese boy with clinical features of CN-I, but without a known pathogenic mutation in *UGT1A1*. This led us to evaluate a novel variant in his *UGT1A1* gene, Val275del, including an expression of a recombinant UGT1A1 that carries the Val275del variant and analyzing its enzyme activity to demonstrate pathogenicity. The identification of this new CN-I variant contributes to our understanding of the function of UGT1A1 specifically, as well as to the UGT family of enzymes in general.

Materials and Methods

The study protocol was approved by the Yale University Institutional Review Board, and written informed consent was obtained from the family. DNA was isolated from saliva samples collected from the patient, parents and all siblings (DNAGenotek, Ottowa, ON). The following PCR oligonucleotides were used for Sanger sequencing:

5’ tttctgtgcgacgtggttta 3’

5’ gggctagttaatcgatccaaa 3’

Mutagenesis and Production of Recombinant UGT1A1 proteins:

Expression of wild type (WT) UGT1A1 in insect cells as a C-terminal His-tagged protein has previously been described (Kurkela et al., 2003). To express the variant protein described in this study, the V275del mutation was introduced in the pFastBac-UGT1A1-XHC (WT) plasmid using QuikChange (Stratagene, LaJolla CA), with the following PCR oligonucleotides:

5’ catcatgcccaatatggtttttggtggaatcaactgcct 3’

5’ aggcagttgattccaccaaaaaccatattgggcatgatg 3’

The entire coding region of the resulting UGT1A1-V275del cDNA was sequenced. Recombinant baculovirus was then used to infect Sf9 insect cells as previously described and crude membrane homogenates of the cells were prepared for the activity assays (Kurkela et al., 2003). Protein concentrations were measured using Pierce BSA Protein Assay Kit (Thermo Scientific, Rockford, IL) and Western blotting was done to confirm proper recombinant protein expression using Tetra-His antibody (QIAGEN, Hilden, Germany) against the C-terminal His-tag.

UGT1A1 Activity Assays:

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Reagents for glucuronidation reactions and analysis were from Sigma-Aldrich (St. Louis, MO) except for estradiol-3-glucuronide, which was from Cayman Chemical (Ann Arbor, MI). Glucuronidation activity assays were conducted in 100 µl total volume. Reactions contained 50 mM sodium phosphate buffer pH 7.4, 10 mM magnesium chloride, 5 mM uridine 5'-diphosphoglucuronic acid (UPDGA) ammonium salt, 1% dimethyl sulfoxide, 80 µg of total protein from recombinant UGT1A1 membrane homogenates and substrate. Reactions were prepared on ice in the absence of UPDGA, incubated for 15 min at +4°C, followed by 5 min at +37°C. Reactions were initiated by addition of UPDGA, and then incubated at +37°C for 15 min (WT with estradiol), 60 min (WT with 4-methylumbelliferone (4-MU)) or 180 min (V275del variant, both substrates). Reactions were terminated by addition of 10 µl of 4 M perchloric acid and placed on ice. Samples were centrifuged 10 min at 16,000g, and the supernatants were used for HPLC analyses.

Glucuronides were quantified using an 1100 HPLC equipped with a fluorescence detector with 120 EC-C18 Poroshell column, 4.6 x 100mm, 2.7 µm (Agilent, Palo Alto, CA). Eluents for chromatography were 0.1% formic acid (A) and acetonitrile (B). Flow rate, column temperature and injection volume were constant at 1 ml/min, 40 °C and 60 µl, respectively. The HPLC run for 4-MU-glucuronide was 0-1 min (15% B), 1-4 min (15->50% B), 4-6 min (50->80% B), 6-9 min (15% B) and the excitation and emission wavelength were set at 318 and 378 nm, respectively. The HPLC run for β-estradiol-3-glucuronide was 0-1 min (25% B), 1-5 min (25->50% B), 5-7 min (50->80% B), 7-10 min (25% B) and the excitation and emission wavelength were set at 222 and 309 nm, respectively. Quantification and identification of 4-MU glucuronide and estradiol-3-glucuronide were based on authentic commercial standards.

Results

The patient presented as a 2-year-old ethnically Sudanese boy who lived in Egypt for the first two years of his life. He was diagnosed with CN-I shortly after birth in the setting of unconjugated hyperbilirubinemia (maximum level was 32 mg/dL) with response to phototherapy and no significant response to phenobarbital. No genetic testing was done at that time. His parents are first cousins and he has five siblings who are all healthy. Prenatal history was notable for maternal hyperthyroidism treated with thiouracil. He immigrated to the United States with his family for further management, including liver transplantation evaluation.

At initial evaluation in the United States, clinical laboratory analysis found the unconjugated bilirubin to be 26.8 mg/dL (see clinical lab results in Supplementary Table S1). Abdominal ultrasound was normal. He had a normal neurological exam and development. He was started on intensive phototherapy with a decrease in unconjugated bilirubin to 9 mg/dL after 30 days. He also received a trial of phenobarbital, though there was no clear benefit from it and this was stopped. At 2 years and 9 months of age, his unconjugated hyperbilirubinemia had risen to 15.2 mg/dL, then gradually increased over the subsequent 2 months to 21.0 mg/dL despite continued phototherapy. He received an orthotopic liver transplant just prior to his third birthday. His jaundice resolved within days and subsequent bilirubin level was <0.2 mg/dL. Pathology of his native liver (Supplementary Figure S1) demonstrated normal histology and no fibrosis on trichrome stain.

As part of the evaluation for clinically-suspected CN-I, the patient underwent clinical *UGT1A1* gene sequencing (Prevention Genetics, Marshfield, WI), which revealed two homozygous variants. One was the common dinucleotide repeat variant $A(TA)_7TAA$ present in

the *UGT1A1* promoter. Although this variant has been associated with Gilbert syndrome, it is present in 40% of the general population and was thus considered nonpathogenic (Bosma et al., 1995). The second was a novel in-frame deletion, p.Val275del (NM_000463.2, c.824_826delTTG), that was not found in any publicly available databases or in the literature and was thus classified as being of uncertain significance. Subsequent targeted Sanger sequencing of the patient, parents and siblings revealed that both parents as well as all three brothers were heterozygous for this variant, whereas both sisters had the reference allele (Figures 1A and 1B). A review of the protein sequence at this locus reveals that this residue varies only between valine or isoleucine across UGT1A1 from multiple species, as well as across all functional human UGTs (Figures 1C and 1D). Since the high-resolution structure of UGT1A1 is not available, we found that within the homology model for the enzyme, Val275 is situated within Nβ7, a distinct β-strand that is located in the N-terminal domain of the enzyme and lies next to the protein's conserved envelope region (Laakkonen and Finel, 2010). We further performed a tertiary structure analysis using the Phyre2 tool, which predicted that the in frame deletion of Val275 may result not only in shortening Nβ7, but of the following α-helix, Cα1, and of the subsequent β-strand, Cβ1 as well (Supplementary Figure S2).

To further assess the functional consequences of the new variant, we expressed the Val275del mutant of human UGT1A1 in baculovirus-infected SF9 insect cells. Western blotting confirmed protein expression of both WT and Val275del proteins (Figure 2A). Glucuronidation assays revealed no detectable activity for the Val275del enzyme with either of the two different validated and highly sensitive UGT1A1 substrates, 4-MU and β-estradiol (Table 2B). This

absence of activity was noted despite higher expression levels of the mutant protein, as seen in Figure 2A, as well as incubation with the mutant protein for three times longer than the WT.

Discussion

This study describes a novel variant in *UGT1A1*, Val275del, in a Sudanese boy with severe unconjugated hyperbilirubinemia. Expansion of the genetic analysis to include the patient's family revealed this phenotype segregated with homozygosity of Val275del. Although the clinical presentation was highly consistent with the diagnosis of CN-I, the fact that this variant had not been previously described prevented both the clinical genetics lab and the care providers from providing the patient's family with a precise diagnosis. We therefore undertook combined genetic and functional studies of this variant to determine its pathogenicity.

The gene locus for *UGT1A* enzyme family consists of a series of unique exons that serve as the first exon for each isoform and provide their substrate specificity via alternative splicing (Gong et al., 2001). Downstream from these, there are four common exons that are identical among each UGT1A family member. This patient's variant, Val275del, was located near the end of the first exon and would therefore be specific to *UGT1A1* alone, the enzyme uniquely involved in bilirubin conjugation, without affecting other UGTs.

When compared to UGT1A1 proteins across multiple species, as well as to all other functional human UGTs, this residue varies only between the highly similar branch-chained aliphatic amino acids valine and isoleucine, further suggesting a crucial role. Interestingly, UGT1A1 is the only UGT1A family member with a valine at this position; the remainder have isoleucine. In contrast, all active UGT2 enzymes have valine at this residue. Furthermore, human UGT1A1 with a valine to isoleucine substitution at this site has previously been shown

to be fully functional (Ciotti et al., 1995). Val275 also lies adjacent to the highly conserved Gly276 residue, that has previously been implicated in cases of CN-I (Ciotti et al., 1995). Additionally, our analysis of secondary structure predicted that Val275 deletion may result in shortening of three consecutive motifs, the β-strands Nβ7 and Cβ1, and the α-helix that lies between them, Cα1. This raises the possibility of altered protein packing. Finally, the fact that this patient's variant was a deletion suggested that it was likely leading to CN-I, as *UGT1A1* coding variants that result in the milder CN-II are known to be caused only by missense mutations within the gene (Kadakol et al., 2000). This also contrasts with Gilbert syndrome, where the molecular defect lies outside the coding region and in the gene's promoter, thereby affecting protein expression levels without altering actual enzyme function (Bosma et al., 1995).

To further examine this new variant, we expressed it alongside WT UGT1A1 protein *in vitro*, and carefully searched for enzyme activity. As summarized in Figure 2B, such activity was easily measured in WT UGT1A1, but not in UGT1A1-Val275del, strongly suggesting that this variant is inactive. The *in vitro* results from the expressed new variant, in combination with the genetic analyses and the consistent presence of valine or isoleucine at this residue across all known active UGTs in humans, strongly support the identification of Val275del as a pathogenic allele leading to CN-I. This result also speaks to the crucial nature of this particular residue in the function of UGT enzymes in general.

Although the discovery of novel alleles in previously described rare diseases is not a new phenomenon, this case is illustrative of a larger deficiency in our knowledge base. A seminal article from late 2016 noted that most patients and controls reported in genomic databases are from either European (81%) or Asian (14%) descent, with only around 3% coming from African

populations (Popejoy and Fullerton, 2016). Therefore, it is not surprising that patients with rare diseases in genomically underrepresented populations may present with novel variants in known genes, as in the case of this child from Africa, or perhaps even variants in totally novel genes. Ethnic variability has been shown specifically for other UGTs, but in a broader context this speaks to the need for greater efforts directed in generating databases from diverse populations in order to refine the interpretation of the significance of such mutations and widen the diversity and inclusion of genomic data (Fowler et al., 2015; Bentley et al., 2017).

In summary, we provide both genetic and functional evidence supporting the identification of the novel Val275del allele of *UGT1A1* as an etiology of CN-I in a Sudanese child. This report expands our understanding of the function of UGT enzymes by revealing a position that appears to be strictly conserved to either one of two residues, Val or Ile. Furthermore, we use this case to emphasize the need for broader diversity and inclusion of various populations in genomic medicine.

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Authorship contribution:

Gathered and analyzed clinical data: Elfar, Iuga, Lobritto, Chan

Performed sequencing and analyzed genetic data: Ji, Sega, Konstantino, Lakhani

Performed and analyzed *in vitro* experiments: Järvinen, Mosorin, Finel

Wrote or contributed to manuscript writing: Elfar, Järvinen, Ji, Mosorin, Sega, Finel, Lakhani

Footnotes:
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Figure Legends:

Figure 1: (A) Sanger sequencing chromatograms demonstrating homozygous reference (top panel, from patient's sister), homozygous V275del (middle panel, from patient) and heterozygous (bottom panel, from patient's father) genotypes. (B) Pedigree showing patient with homozygous V275del as black box; heterozygous individuals are noted in gray and homozygous reference individuals in white. (C) Multiple species alignment of UGT1A1 protein sequence showing the region containing Val275. Residues with amino acids identical to the human sequence are highlighted in yellow. (D) Aligned sequence of all functional human UGTs in the region of Val275 of UGT1A1. Color codes of residues indicate nature of side chains: red = small + hydrophobic (A, V, F, P, M, I, L, W), blue = acidic (D, E), magenta = basic (R, K), green = hydroxyl + sulfhydryl + amine + G (S, T, Y, H, C, N, G, Q).

Figure 2: (A) Western blot showing normal migration of V275del and wild type (WT) UGT1A1 proteins expressed in Sf9 cells. (B) Table of glucuronidation enzyme activity of WT and V275del proteins (n=3 in triplicates). 4-MU = 4-methylumbelliferone.

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Figure 1

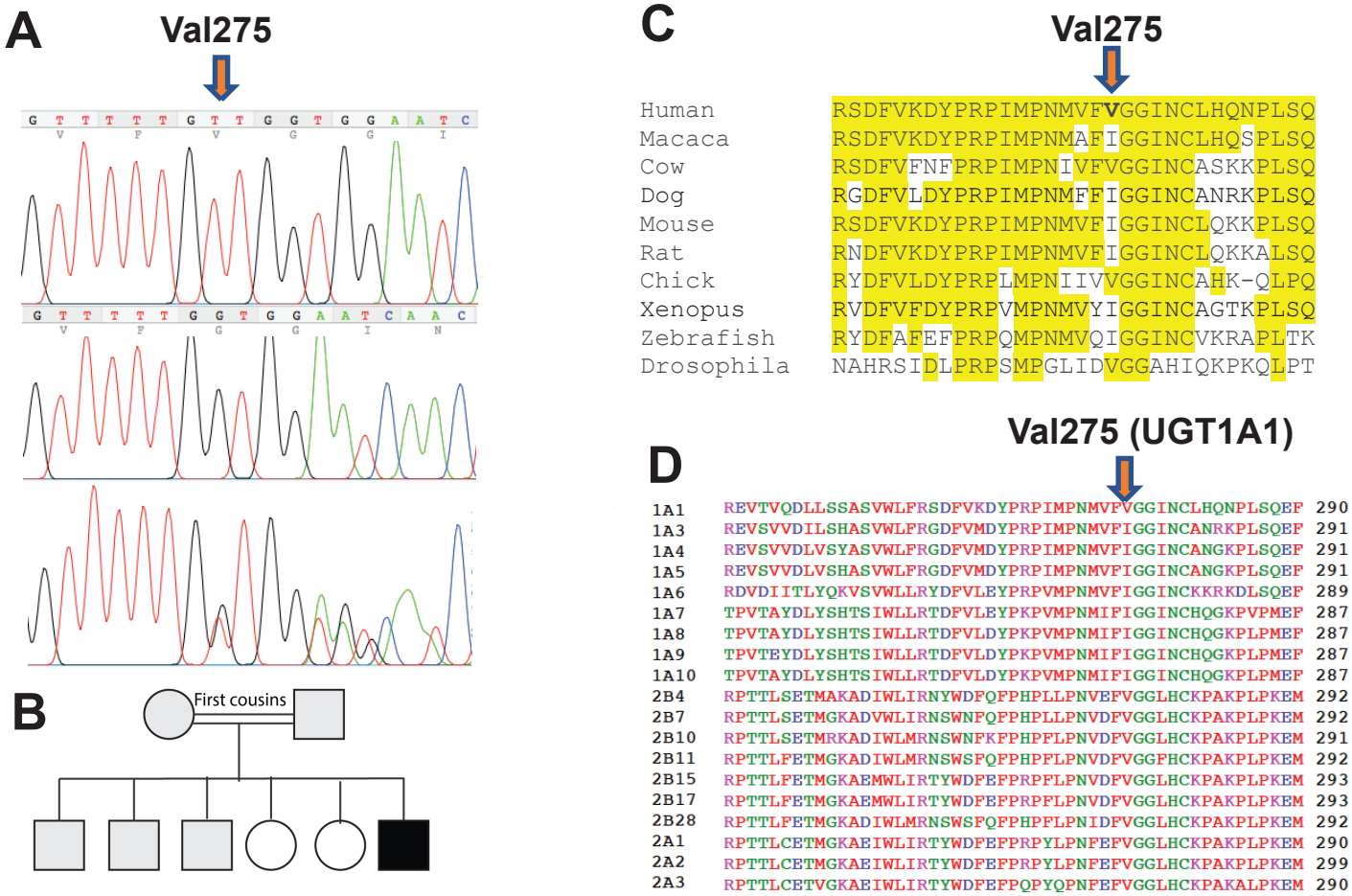
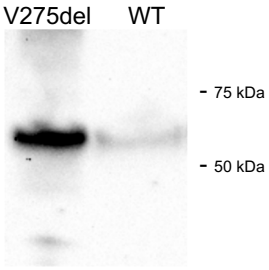


Figure 2

A



B

Substrate	UGT1A1 pmol/mg/min ± S.D.	UGT1A1-V275del
4-MU, 25 μM	70 ± 2	Not detected
4-MU, 250 μM	453 ± 12	Not detected
β-estradiol, 10 μM	100 ± 9	Not detected
β-estradiol, 100 μM	344 ± 21	Not detected

Supplemental Data for

A Novel Pathogenic *UGT1A1* Variant in a Sudanese Child with Type I Crigler-Najjar Syndrome

(Submission to journal: Drug Metabolism and Disposition)

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WE and EJ contributed equally to this work.

MF and SAL contributed equally to this work.

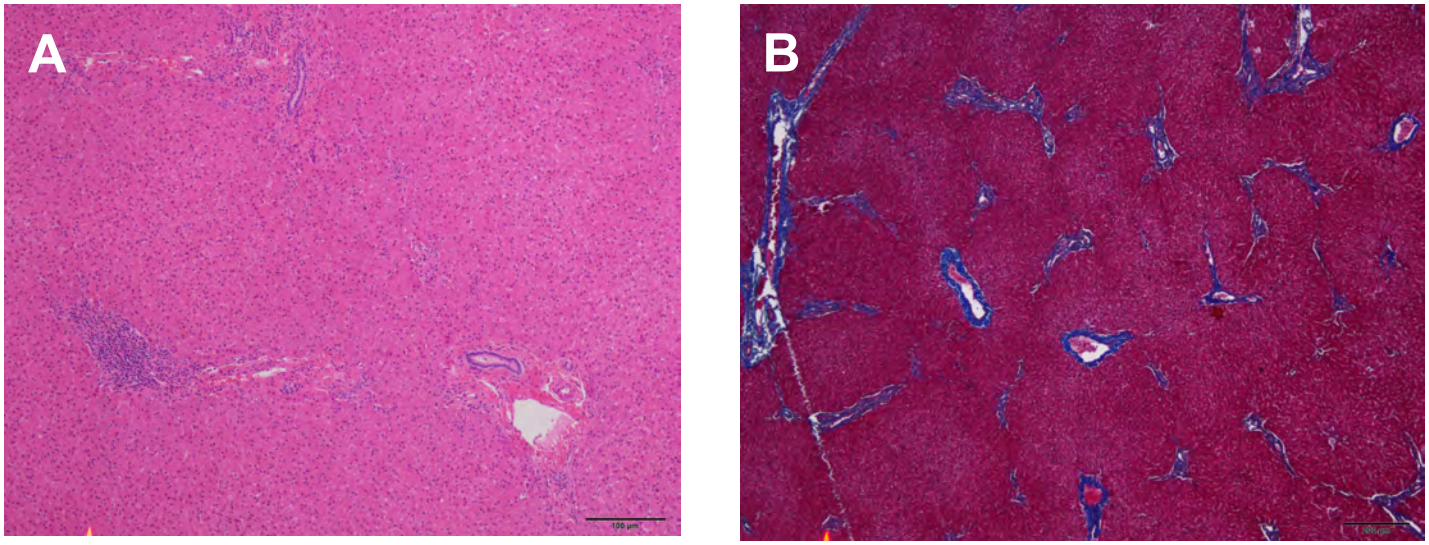
Primary laboratory of Origin: Pediatric Genomics Discovery Program, Department of Pediatrics, Yale University School of Medicine, USA and Division of Pharmaceutical Chemistry and Technology, University of Helsinki, Finland

Supplementary Table S1

Test name	Patient value	Normal range
WBC	9.1	*5.0 - 17.0 x10^3/μL*
RBC	5.0	*3.9 - 5.3 x10^6/μL*
Hemoglobin	11.6	*11.5 - 14.0 g/dL*
Platelets	366	*150 - 330 x10^3/μL*
Sodium	142	*133 - 145 mmol/L*
Potassium	4.8	*3.6 - 5.2 mmol/L*
Chloride	103	*96 - 108 mmol/L*
CO2	24	*20 - 28 mmol/L*
BUN	21	*6 - 20 mg/dL*
Creatinine	0.48	*0.30 - 0.70 mg/dL*
Albumin	4.7	*3.5 - 5.2 g/dL*
Bilirubin (total)	27.0	*0.0 - 1.2 mg/dL*
Bilirubin (conjugated)	0.2	*0.0 - 0.3 mg/dL*
Bilirubin (unconjugated)	26.8	*0.1 - 1.0 mg/dL*
Alkaline Phosphatase	281	*145 - 320 U/L*
AST	58	*0 - 50 U/L*
ALT	32	*0 - 50 U/L*
Protime	10.6	*9.2 - 12.3 sec*
aPTT	31.3	*25.8 - 37.9 sec*
Retic %	1.0	*1.0 - 1.8 %*
GGT	15	*8 - 61 U/L*
CRP	<1	*0 - 10 mg/L*
TSH	2.24	*0.85 - 6.50 μIU/mL*
Free T4	1.3	*0.9 - 1.7 ng/dL*

Supplementary Table S1:
Patient's lab values from initial evaluation in the United States at 2 years of age, as performed at a certified clinical laboratory.

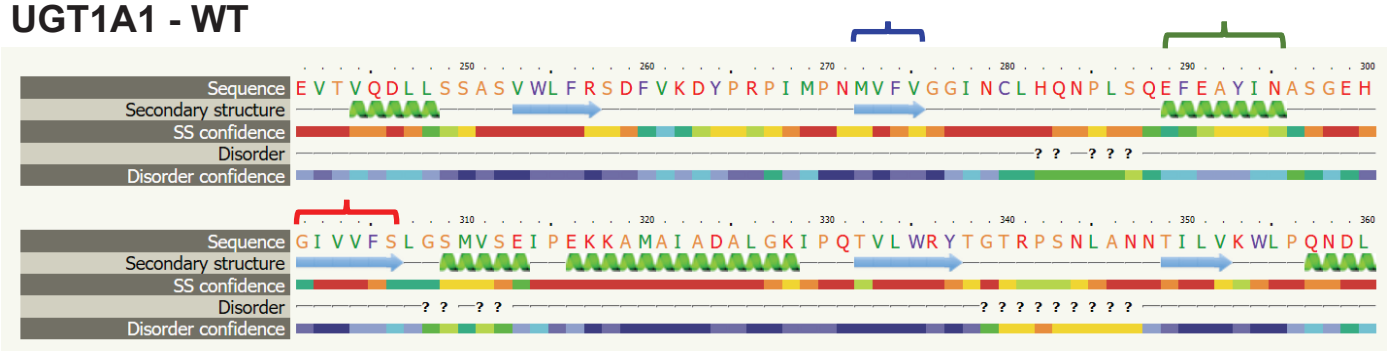
Supplementary Figure S1



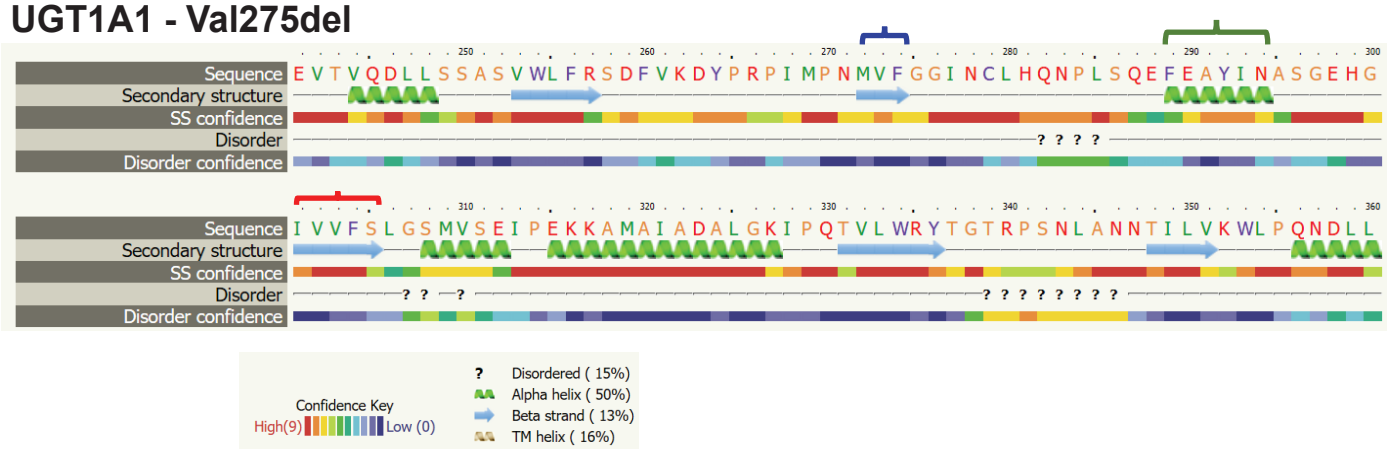
Supplementary Figure S1: (A) Hematoxylin & eosin and (B) trichrome staining of native liver, demonstrating normal architecture with no evidence of fibrosis.

Supplementary Figure S2

UGT1A1 - WT



UGT1A1 - Val275del



Supplementary Figure S2: Effects of Val275del on the secondary structure of UGT1A1 using Phyre2 (Kelley LA, et al. "The Phyre2 web portal for protein modeling, prediction and analysis." Nat Protoc 10(6): 845-858, 2015).
The region around Val275 is shown for wild type UGT1A1 (top) and the Val275del variant (bottom). The model predicts the shortening of the beta strand containing Val275, N-beta-7 (blue bracket), the following alpha-helix, C-alpha-1 (green bracket), and of the following beta-strand, C-beta-1 (red bracket).