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Spectrum of *UGT1A1* variants in Pakistani children affected with inherited unconjugated hyperbilirubinemias

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ABSTRACT

Inherited unconjugated hyperbilirubinemias are a group of disorders characterized by increased levels of serum unconjugated bilirubin and arise because of the imbalance between its production and elimination from the body. It includes Crigler-Najjar syndrome and Gilbert syndrome. Crigler-Najjar syndrome type 1 represents the extreme severe end of the spectrum with complete absence of hepatic bilirubin uridine diphosphoglucuronate glucuronosyltransferase (UGT1A1). Crigler-Najjar syndrome type 2 patients have intermediate levels of bilirubin owing to incomplete deficiency of UGT1A1, and Gilbert syndrome lies at the extreme mild end of the spectrum with only slightly raised bilirubin level. Here, we present spectrum of *UGT1A1* genetic variants in 25 Pakistani children from 23 unrelated families affected with persistent unconjugated hyperbilirubinemias. The promoter region, coding exons and splice junctions of the *UGT1A1* were PCR amplified and subjected to Sanger sequencing. Eleven sequence variants were identified underlying disease phenotype including a novel c.582delC variant. Overall, c.622_625dupCAGC was the most frequent variant followed by c.1021C > T found in Crigler-Najjar syndrome type 1 patients. The evaluation of promoter polymorphism $A(TA)_nTAA$ in the affected children and their families further supported the body of evidence that the $A(TA)_7TAA$ allele could enhance the effect of other structural variants in Crigler-Najjar syndrome patients. To our knowledge, this is the first comprehensive study on molecular genetics of persistent unconjugated hyperbilirubinemias from Pakistan. This study expands the spectrum of *UGT1A1* variants and should help in improved clinical diagnosis, genetic counseling and prenatal diagnosis of the affected families.

1. Introduction

Bilirubin is the final product resulting from catabolism of haem, the clearance of which is primarily attributable to liver. Inherited anomalies in liver uptake of bilirubin, conjugation, or transport result in its systemic accumulation leading to jaundice as the first symptom. Inherited unconjugated hyperbilirubinemias include Crigler-Najjar syndrome type 1 (CN1), Crigler-Najjar syndrome type 2 (CN2) and Gilbert syndrome (GS). CN syndrome is inherited in autosomal recessive manner although CN2 has shown autosomal dominant inheritance in few cases [1,2]. Among these, CN1 is the most severe phenotype followed by CN2 and Gilbert syndrome, respectively. The clinical presentation of CN2 and GS sometimes overlap making it difficult to distinguish the two phenotypes.

The molecular etiology of these disorders lies in the conjugation of bilirubin, which is attributable to the enzyme uridine-diphospho glucuronosyl transferase 1A1 encoded by *UGT1A1* [3–6]. Any genetic lesion in the *UGT1A1* or its promoter that results in reduced expression/activity of the enzyme leads to one of these phenotypes depending on the extent to which the enzyme expression or activity is compromised. CN1 is identified with nearly or complete absence of UGT1A1 activity and hence highest serum unconjugated bilirubin levels (340–850 μmol/L). It clinically presents with kernicterus often accompanied with lethargy and reduced muscle tone. CN1 shows non-compliance to phenobarbital therapy and the only left-over treatment is liver transplantation [7]. Most of the CN1 patients die in childhood due to kernicterus and associated neurological problems. CN2 patients are often observed with intermediate levels of serum unconjugated bilirubin (85–340 μmol/L) consistent with the moderate reduction in enzyme activity, the restoration of which is possible with phenobarbital administration. They are less likely to develop kernicterus and most of them survive to adulthood [8]. GS patients have only mild elevation of serum unconjugated bilirubin levels often without clinical symptoms except after administration of drugs, which are metabolized by UGT1A1

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[9].

The current study was aimed at identification of the spectrum of *UGT1A1* sequence variants in Pakistani children affected with inherited unconjugated hyperbilirubinemias and expands our previous work reported in 2013 [10].

2. Methodology

The study was approved by institutional review board of Quaid-i-Azam University, Islamabad. Twenty-five children (from 23 unrelated families) affected with persistent unconjugated hyperbilirubinemias were recruited from Punjab (23) and Khyber Pakhtunkhwa (2) Provinces of Pakistan. They were referred to our Medical Genetics Research Laboratory for molecular investigations from the Department of Pediatric Gastroenterology of the Children's Hospital & the Institute of Child Health Lahore, the Holy Family Hospital Rawalpindi and the Khyber Teaching Hospital Peshawar. Seventeen children were diagnosed with CN1, six with CN2, and two with GS. Informed consent was obtained from guardians of all probands for enrollment in the current study. The clinical data collected from the affected children were analyzed using SPSS version 20. The numerical data including values of serum bilirubin, serum direct bilirubin, alanine aminotransferase (ALT), prothrombin time (PT) and serum albumin were presented as mean ± SD. The subtypes of CN1 & CN2 were compared using Fisher's Exact Test. *P*-values < .05 were considered as significant.

Peripheral blood samples were collected from the affected children and their parents where available. DNA was isolated using Wizard Genomic DNA Purification Kit (Promega Corporation) followed by quantification using QuantiFluor Dye Systems on Quantus Fluorometer (Promega Corporation) and diluted to 100 ng/μl for amplification by polymerase chain reaction. The promoter region and all five exons including splice junctions of the *UGT1A1* were PCR amplified from the genomic DNA using primers from flanking regions (sequences are available upon request). The PCR was carried out in a thermocycler (Labnet International) in 50 μL reaction volume containing 25uL Quick-Load Taq 2× Master Mix (NEB), 100 ng genomic DNA, and 50pmoles of each primer using the following parameters: initial denaturation at 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 35 s, followed by final extension at 72 °C for 4 min. The PCR products were purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific) and subjected to direct DNA sequencing using BigDye Terminator Cycle Sequencing Kit v3.1 (Thermo Fisher Scientific). The sequencing products were purified by ethanol precipitation and were sequenced in an ABI 3730xl automated sequencer. Sequence chromatograms obtained were aligned using BioEdit sequence alignment editor version 7.0.5.3 [11].

3. Results

A cohort of 25 children affected with persistent unconjugated nonhemolytic hyperbilirubinemia was enrolled in the current study. All children were born to consanguineous marriages except CNS-30. The patients were classified as CN1 (17), CN2 (6) or Gilbert syndrome (2). The serological assays for HBV and HCV were negative in all patients. Hematological and biochemical investigations did not reveal any evidence of hemolysis, and liver function tests other than bilirubin were normal.

The mean total bilirubin level for CN1 patients was 528.73 ± 130.13 μmol/L and was significantly different (Fisher's Exact test) from CN2 patients for which the mean total bilirubin was 162.96 ± 87.21 μmol/L. The analysis revealed that a significant proportion of CN1 patients (11/17) experienced fits and exchange transfusion. However, the differences in other parameters i.e., consanguinity, family history, developmental delay, direct bilirubin, ALT, PT, and albumin were found insignificant (Table 1).

3.1. Mutation screening of the UGT1A1

Mutation screening of *UGT1A1* in all affected children was performed by direct sequencing of five coding exons, splice junctions and promoter region at 5′ end till 200 bp. Eleven sequence variants were identified including a novel variant p.Arg195GlyfsTer10 summarized in Tables 2 and 3. The variants were distributed over the entire coding region (Fig. 1).

3.2. Children affected with CN1

Seventeen children were clinically diagnosed with CN1. Seven homozygous *UGT1A1* variants were identified (Fig. 2) including two missense (p.Gly276Arg, p.Pro387His), one nonsense (p.Arg341Ter) and four frameshift variants (p.Gly119AspfsTer28, p.Arg195GlyfsTer10, p.Arg209ProfsTer50, p.Asn348ThrfsTer18). One of these variants (p.Arg195GlyfsTer10) was novel while others have been previously reported [10,12–15]. In our study, p.Arg209ProfsTer50 was most frequently observed variant identified in five (29.4%) CN1 patients (CNS-12, CNS-20, CNS-22, CNS-25, CNS-30) followed by p.Arg341Ter identified in four (23.5%) CN1 patients (CNS-8, CNS-23, CNS-26, CNS-29). Three variants were identified in two (11.7%) CN1 patients apiece: p.Arg195GlyfsTer10 (CNS-21A, CNS-21B), p.Gly276Arg (CNS-18, CNS-19) and p.Asn348ThrfsTer18 (CNS-11, CNS-27). Each of the two other variants were identified once (5.8%) in our study: p.Gly119AspfsTer28 (CNS-10) and p.Pro387His (CNS-14).

3.3. Novel CN1 variant

A novel homozygous single base pair deletion p.Arg195GlyfsTer10 was identified in two siblings of the same CN1 family (CNS-21A, CNS-21B). It is localized in the aglycone binding domain of UGT1A1 and predicts a frameshift and premature truncation at amino acid position 204 (p.Arg195GlyfsTer10).

3.4. Children affected with CN2

Six children were clinically diagnosed with CN2 and screened for *UGT1A1* sequence variants. Five variants were identified (Fig. 3) including four missense variants (p.Gly71Arg, p.Arg108Cys, p.Ile322Val, p.Tyr486Asp) and one nonsense variant (p.Arg341Ter). These variants have been previously reported [16–18].

Two affected children were compound heterozygous for different combinations of three sequence variants: p.Gly71Arg & p.Tyr486Asp (CNS-28) and p.Arg341Ter & p.Tyr486Asp (CNS-17). One affected child (CNS-15) was homozygous for a missense variant p.Arg108Cys in concurrence with homozygous promoter polymorphism $A(TA)_7TAA$. CNS-13 was heterozygous for a missense variant p.Ile322Val and promoter polymorphism $A(TA)_7TAA$. These alleles segregated in cis from the maternal chromosome as she was heterozygous for both alleles. In CNS-24 and CNS16, no variant was identified (in the coding exons and splice junctions of *UGT1A1*) except promoter polymorphism $A(TA)_7TAA$ allele in heterozygous condition. Clinically, the patients were not different from other patients in CN2 cohort. Hence, there might be other variants in the intronic or regulatory regions [19].

p.Ile322Val has been previously reported by Farheen et al. in compound heterozygous state with a promoter variant in Gilbert syndrome patient. However, the functional analysis has not been performed [18]. The variant was found in gnomAD with “deleterious effect” predicted by SIFT and a frequency of 0.0145 in South Asian population with only six homozygotes. The in-silico prediction using SIFT and Polyphen predicted the allele as ‘deleterious (score 0.04)’ and ‘possibly damaging (score 0.76)’, respectively, while PROVEAN predicted the effect of allele as “neutral”. The Grantham distance between the two amino acids is 29 lying in conservative range. Hence, the allele could be classified as “variant of uncertain significance” but may

Table 1
Comparison of biochemical investigations between CN1 and CN2 affected children.

	CN1 affected children (n = 17)	CN2 affected children (n = 6)	P value (Fischer's exact)	Significance
	Mean ± SD	Mean ± SD		
Bilirubin (μmol/L)	528.73 ± 130.13	162.96 ± 87.21	< 0.05	Significant
Direct Bilirubin(μmol/L)	21.89 ± 17.78	9.41 + 2.39	0.11	Insignificant
ALT (IU/mL)	37.87 ± 25.47	19.00 ± 6.36	0.09	Insignificant
PT (seconds)	14.75 ± 2.76	14.50 ± 2.12	0.90	Insignificant
Albumin (g/L)	0.035 ± 0.006	0.0372 ± 0.0034	0.45	Insignificant

ALT; Alanine aminotransferase, PT; Prothrombin time.

Table 2
UGT1A1 variants identified in children affected with CN1.

	Patient ID	Exon	Homozygous variants	Predicted protein variant	Reference	Promoter polymorphism (TA) allele
1	CNS-8	3	c.1021C > T	p.Arg341Ter	10	$A(TA)_6TAA$/ $A(TA)_6TAA$
2	CNS-10	1	c.353dupA	p.Gly119AspfsTer28	12	$A(TA)_7TAA$/ $A(TA)_7TAA$
3	CNS-11	3	c.1043delA	p.Asn348ThrfsTer18	13	$A(TA)_6TAA$/ $A(TA)_6TAA$
4	CNS-12	1	c.622_625dupCAGC	p.Arg209ProfsTer50	10	$A(TA)_6TAA$/ $A(TA)_6TAA$
5	CNS-14	4	c.1160C > A	p.Pro387His	14	$A(TA)_7TAA$/ $A(TA)_7TAA$
6	CNS-18	1	c.826G > C	p.Gly276Arg	16	$A(TA)_6TAA$/ $A(TA)_6TAA$
7	CNS-19	1	c.826G > C	p.Gly276Arg	16	$A(TA)_6TAA$/ $A(TA)_6TAA$
8	CNS-20	1	c.622_625dupCAGC	p.Arg209ProfsTer50	10	$A(TA)_6TAA$/ $A(TA)_6TAA$
9	CNS-21A	1	c.582delC	p.Arg195GlyfsTer10	This study	$A(TA)_6TAA$/ $A(TA)_6TAA$
10	CNS-21B	1	c.582delC	p.Arg195GlyfsTer10		$A(TA)_6TAA$/ $A(TA)_6TAA$
11	CNS-22	1	c.622_625dupCAGC	p.Arg209ProfsTer50	10	$A(TA)_6TAA$/ $A(TA)_6TAA$
12	CNS-23	3	c.1021C > T	p.Arg341Ter	10	$A(TA)_6TAA$/ $A(TA)_6TAA$
13	CNS-25	1	c.622_625dupCAGC	p.Arg209ProfsTer50	10	$A(TA)_6TAA$/ $A(TA)_6TAA$
14	CNS-26	3	c.1021C > T	p.Arg341Ter	10	$A(TA)_6TAA$/ $A(TA)_6TAA$
15	CNS-27	3	c.1043delA	p.Asn348ThrfsTer18	13	$A(TA)_6TAA$/ $A(TA)_6TAA$
16	CNS-29	3	c.1021C > T	p.Arg341Ter	10	$A(TA)_6TAA$/ $A(TA)_6TAA$
17	CNS-30	1	c.622_625dupCAGC	p.Arg209ProfsTer50	10	$A(TA)_6TAA$/ $A(TA)_6TAA$

Table 3
UGT1A1 variants identified in children affected with CN2 and GS.

	Diagnosis	Patient ID	Exon	Variants	Predicted protein variants	References	Promoter polymorphism (TA) alleles
1	CN2	CNS-13	2	c.964A > G	p.Ile322Val	18	$A(TA)_6TAA/A(TA)_7TAA$
2	CN2	CNS-15	1	c.322C > T	p.Arg108Cys	10	$A(TA)_7TAA/A(TA)_7TAA$
			1	c.322C > T	p.Arg108Cys		
3	CN2	CNS-16	Promoter			20	$A(TA)_6TAA/A(TA)_7TAA$
4	CN2	CNS-17	3	c.1021C > T	p.Arg341Ter	10,15	$A(TA)_6TAA/A(TA)_6TAA$
			5	c.1456T > G	p.Tyr486Asp		
5	CN2	CNS-24	Promoter			20	$A(TA)_6TAA/A(TA)_7TAA$
6	CN2	CNS-28	5	c.1456T > G	p.Tyr486Asp	15	$A(TA)_6TAA/A(TA)_6TAA$
			1	c.211G > A	p.Gly71Arg		
7	GS	CNS-9A	Promoter			20	$A(TA)_7TAA/A(TA)_7TAA$
8	GS	CNS-9B	Promoter			20	$A(TA)_7TAA/A(TA)_7TAA$

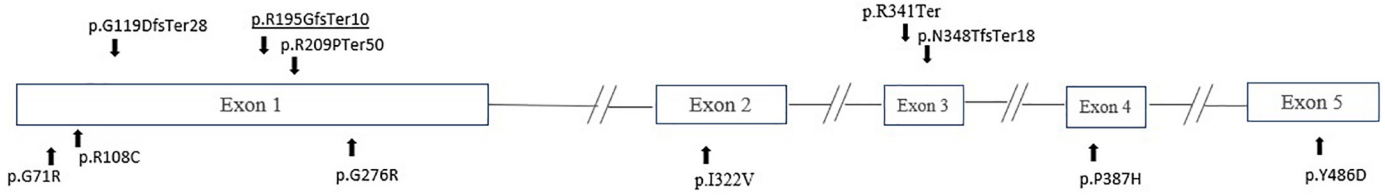

Fig. 1. *UGT1A1* variants identified in this study in Pakistani children affected with Crigler-Najjar syndrome. Variants are distributed over all exons of *UGT1A1*. Novel variants are underlined. Promoter region is not shown.

potentially be pathogenic or (benign).

3.5. GS

A family affected with the GS was investigated for *UGT1A1* variants. In the family, two affected (CNS-9A & CNS-9B) and two normal siblings were born to a consanguineous couple. The patients were homozygous for $A(TA)_7TAA$ allele while both parents and two healthy siblings were heterozygous [$A(TA)_6TAA/A(TA)_7TAA$].

3.6. Association of $A(TA)_7TAA$ polymorphism with UGT1A1 variants

The role of $A(TA)_7TAA$ promoter polymorphism has been already established with Gilbert syndrome phenotype and reduction of UGT1A1 expression [20]. Thus, all affected children enrolled in the current study were analyzed for this polymorphism as well. In CN1 cohort, two

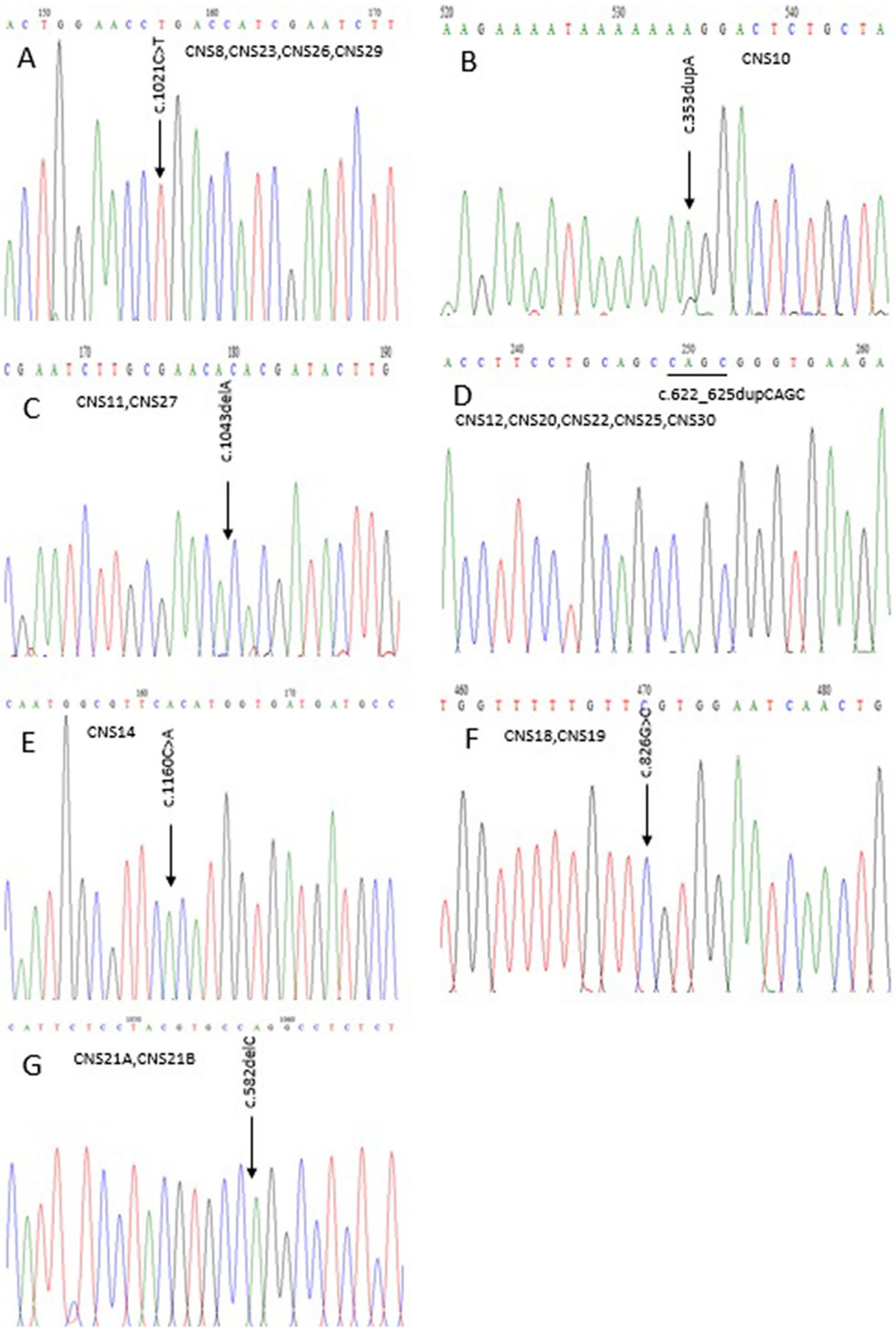

Fig. 2. Variants identified in Crigler-Najjar syndrome type 1 patients. (A) c.1021C > T. (B) c.353dupA. (C) c.1043delA. (D) c.622_625dupCAGC. (E) c.1160C > A. (F) c.826G > C (G) c.582delC. Arrows indicate the positions of variants and line indicates the duplicated nucleotides.

children were found homozygous for $A(TA)_7TAA$ allele while no heterozygote was identified. In CN2 cohort, one child was found homozygous for $A(TA)_7TAA$ allele, three were heterozygous [$A(TA)_6TAA$/$A(TA)_7TAA$] and two were homozygous for wild type $A(TA)_6TAA$ allele (Tables 2 and 3). In both cohorts, the $A(TA)_7TAA$ allele was always present along with another pathogenic allele except in CNS16 and CNS-24 that were heterozygous [$A(TA)_6TAA/A(TA)_7TAA$].

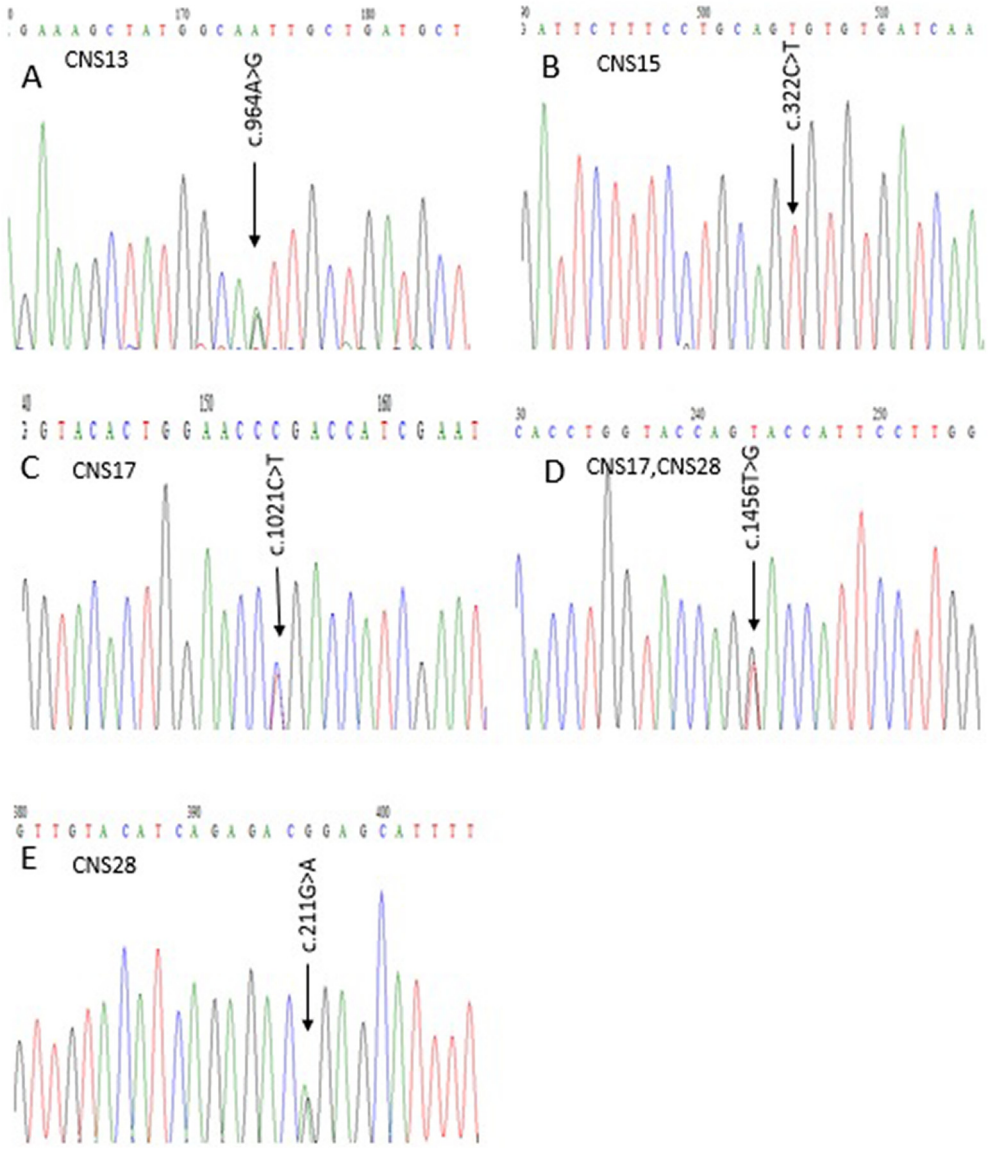

Fig. 3. Variants identified in Crigler-Najjar syndrome type 2 patients. (A) c.964A > G. (B) c.322C > T. (C) c.1021C > T. (D) c.1456T > G. (E) c.211G > A. Arrows indicate the positions of variants.

4. Discussion

A cohort of 25 children affected with persistent unconjugated hyperbilirubinemias were analyzed in the current study to identify the spectrum of *UGT1A1* variants in the local population. The subjects were screened for promoter region, coding exons and splice junctions of *UGT1A1*. Eleven different sequence variants were identified underlying the disease phenotype including six missense, four frameshift, one nonsense, and a promoter polymorphism A(TA)$_7$TAA allele (Fig. 1, Tables 2 and 3).

The identified variants were distributed over the entire *UGT1A1* predominantly in exon 1 corresponding to aglycone binding domain of the enzyme followed by exon 3 (Fig. 1). In our study, all CN1 affected children were found homozygous for their respective mutant alleles: 14/17 had truncating variants and 3/17 had missense alterations of critical amino acid residues that abolish or reduce the activity of UGT1A1 enzyme significantly [14,15]. This observation is supported by the previous studies demonstrating the association of truncating mutations with CN1 [19]. Unlike CN1, only 1/6 CN2 patients was homozygous for the mutant allele (p.Arg108Cys) in the coding regions and only one truncating variant (p.Arg341Ter) was observed in a patient (in heterozygous condition) (Table 3).

This study indicates that overall, two *UGT1A1* variants are most prevalent in children affected with CN from Pakistan: p.Arg209ProfsTer50 (10/48 chromosomes, 20.8%) followed by p.Arg341Ter (9/48 chromosomes, 18.8%). The prevalence of p.Arg209ProfsTer50 and p.Arg341Ter within CN1 cohort is 29.4% (10/34 chromosomes) and 23.5% (08/34 chromosomes), respectively. The prevalence data is supported by our previous report [10].

Among 11 variants identified in the current study, one frameshift variant was novel thus expanding the spectrum of known *UGT1A1* variants (www.hgmd.cf.ac.uk). This novel variant p.Arg195GlyfsTer10 is localized in exon 1 corresponding to the aglycone binding domain of UGT1A1. It predicts a premature truncation with a polypeptide length of 203 amino acids of otherwise 533 amino acids thus lacking the carboxy terminal domain with complete absence of glucuronic acid binding domain. The variant might lead to nonsense-mediated mRNA decay and if escaped the protein would lack major portion of the functional enzyme thus rendering it nonfunctional.

In CNS-13, heterozygous alleles (p.Ile322Val and promoter polymorphism A(TA)$_7$TAA) were identified in cis configuration. Farheen et al. reported p.Ile322Val variant in compound heterozygous state in a

GS patient [18] but its occurrence with another heterozygous variant in cis in the current study may not explain the disease phenotype in autosomal recessive pattern of inheritance. The dominant negative effect of UGT1A1 p.Ile322Val allele is also ruled out as the mother of the patient was not affected [1,21,22]. Similarly, two patients CNS16 and CNS24 were identified with a single allele of promoter polymorphism $A(TA)_7TAA$ that could not explain the disease phenotype. Therefore, genetic cause of three patients CNS-13, CNS-16, and CNS-24 in the current study remain unexplained intimating possible involvement of other unidentified factors.

The $A(TA)_7TAA$ allele was observed in higher frequency in CN2 cohort in the presence of another structural variant thus suggesting that the promoter polymorphism plays a role in enhancing the effect of another associated variant [20].

5. Conclusion

This is the first comprehensive study to investigate the spectrum of *UGT1A1* variants in Pakistani children affected with persistent unconjugated hyperbilirubinemias. We found p.Arg209ProfsTer50 and p.Arg341Ter as the most prevalent variants and therefore suggest their screening to rule out early diagnosis of persistent unconjugated hyperbilirubinemias. The study also extends the global mutation spectrum of *UGT1A1* with identification of a novel variant providing bases to explore further insight into the disease biology of hyperbilirubinemias. The data should be useful for genetic counseling of the affected individuals and their families, and prenatal diagnosis.

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Declarations of interest

None.

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Conflict of interests

The authors declare that there is no conflict of interest.

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