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ORIGINAL ARTICLE

A high frequency of Gilbert syndrome (*UGT1A1*28/*28*) and associated hyperbilirubinemia but not cholelithiasis in adolescent and adult north Indian patients with transfusion-dependent β-thalassemia

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Abstract

Hyperbilirubinemia and pigment gallstones are frequent complications in transfusion-dependent β-thalassemia (TDβT) patients. Bilirubin production and clearance are determined by genetic as well as environmental variables like ineffective erythropoiesis, hemolysis, infection-induced hepatic injury, and drug- or iron-related toxicities. We studied the frequency of the Gilbert syndrome (GS), a common hereditary cause of hyperbilirubinemia in 102 TDβT patients aged 13–43 years (median 26 years). Total and unconjugated hyperbilirubinemia were frequent (81.4% and 84.3% patients respectively). Twenty (19.6%) patients showed total bilirubin > 3.0 mg/dL; 53 (51.9%) had an elevation of either alanine or aspartate aminotransferase, or alkaline phosphatase liver enzymes. Nineteen (18.6% of the 92 tested) were positive for hepatitis B or C, or HIV. The mean total and unconjugated bilirubin levels and AST, ALT, and ALP levels in patients positive for hepatitis B or C were not significantly different from negative cases. Eighteen patients (17.7%) had GS: homozygous (TA)7/7 *UGT1A1* promoter motif (the *28/*28 genotype), 48 (47.1%) were heterozygous (TA)6/7. Total + unconjugated bilirubin rose significantly with the (TA)7 allele dose. Fourteen (13.7%) patients had gallstones. There was no significant difference in total/unconjugated bilirubin in patients with/without gallstones and no significant differences in frequencies of gallstones within the three *UGT1A1* genotypes. This largest study in Indian TDβT patients suggests that GS should be excluded in TDβT cases where jaundice remains unexplained after treatable causes like infections, chelator toxicity, or transfusion-related hemolysis are excluded. GS was not associated with gallstones, possibly due to a lower incidence of cholelithiasis overall, a younger age cohort, or other environmental factors.

Keywords Bilirubin · Gallstones · Gilbert syndrome · Jaundice · Liver disease · Thalassemia

Introduction

Thalassemias represent the commonest autosomal recessive disorders worldwide. They are characterized by reduced biosynthesis of one or more globin chain subunits of the hemoglobin tetramer, resulting in severe anemia in homozygous/compound heterozygous states. Classified according to the specific globin chain affected into α, β, δβ,

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γδβ, and other subtypes, β-thalassemia is clinically and epidemiologically the most significant of these disorders in southern Europe, the middle-East, and south Asia [1, 2].

Clinical manifestations of the β-thalassemia syndromes are diverse, arising from variable severities of the primary biosynthetic defects in the β-globin gene (*HBB*) as well as several co-inherited phenotype modifiers. Prominent modulating factors include genetic propensities for high fetal hemoglobin levels and variations in the number and function of α-globin genes co-inherited [1, 2]. Thalassemic patients also suffer from multi-system complications including cardiac dysfunction, delayed growth and sexual maturation, diabetes mellitus, bone disease, transfusion-transmitted infections, and thromboses that are determined by both environmental and genetic modifiers [1, 2]. Hyperbilirubinemia and pigment gallstones are frequent complications with incidences of 15–28% in different studies [3–7]. Cholelithiasis is more frequent in older thalassemics requiring more frequent transfusions. Prevalence peaks in the fourth decade of life [3–6].

Both bilirubin production and clearance are genetically determined processes displaying marked variability in bilirubin glucuronidation capacity among individuals [8–10]. In transfusion-dependent β-thalassemia (TDβT) patients, this is compounded by the variable degrees of ineffective erythropoiesis, destruction of transfused red cells, hepatic dysfunction due to iron overload, drug toxicities or infections, etc. [1–7].

Hepatic clearance of bilirubin is also influenced by the Gilbert syndrome (GS), a variably common hereditary condition characterized by intermittent unconjugated hyperbilirubinemia without hepatocellular disease or hemolysis [9]. GS results from homozygous (TA) repeat expansion in the *UGT1* gene promoter (the *UGT1A1*28/*28* genotype, rs3064744) that encodes the A1 isoform of uridine diphosphate glucuronosyltransferase-1 enzyme. UGT1A1 activity is reduced to 30% of normal in GS patients. A reduction in the liver's ability to conjugate bilirubin leads to increased serum unconjugated bilirubin levels [8–10].

Although largely a benign medical condition not requiring specific treatment, GS in combination with hemolytic anemias like thalassemia, G6PD-deficiency, and hereditary spherocytosis may potentiate severe hyperbilirubinemia and cholelithiasis [3, 5, 6, 10–13]. Its recognition in TDβT is important since subtle unexplained hyperbilirubinemia in these patients may indicate alloimmunization, early/undiagnosed infections, chelating drug toxicities, thyrotoxicosis, etc. leading to potentially unwarranted investigations [1, 2].

Densely populous South Asia accounts for approximately a quarter of the world's population [14]; however, there is only one prior study from this region that evaluates the role of GS in influencing the risk of unconjugated hyperbilirubinemia and cholelithiasis in TDβT patients [11]. We assessed bilirubin levels along with the presence of gallstones and correlated these with the *UGT1A* promoter genotype, the β-globin gene mutational status, and other disease modifiers (Xmn1-$^{G}\gamma$ polymorphism, deletional α-thalassemia, α-triplications) to clarify relationships of these genetic determinants, if any, with clinical and biochemical phenotypes in our cohort of transfusion-dependent β-thalassemics.

Materials and methods

This prospective clinical and laboratory-based study was conducted from January 2017 to June 2018 at a large, state-funded, tertiary-care teaching hospital and research institute. Ethics clearance was obtained and informed consent taken from all patients and/or their guardians. All tenets of the Declaration of Helsinki were followed.

A total of 102 transfusion-dependent β-thalassemia (TDβT) patients aged 13 years or older were enrolled after a detailed history and clinical examination. DNA was extracted from peripheral blood mononuclear cells and its quality was checked spectrophotometrically. *UGT1A1* promoter region was amplified using specific primers (forward: 5′GCCAGTTCAACTGTTGTTGCC3′; reverse: 5′CCACTGGGATCAACAGTATCT3′) [10]. Automated capillary sequencing was done on an ABI 3130 Genetic Analyzer (Applied Biosystems, now ThermoFisher Scientific, Waltham, MA).

Serum bilirubin levels (total and unconjugated), serum alanine and aspartate aminotransferases (ALT and AST), alkaline phosphatase (ALP), and total protein and albumin were estimated using an AU2700 chemistry analyzer (Beckman Coulter Inc., FL). Viral markers (HIV, HBsAg, HCV) were noted from clinical records, as available. These are usually tested for annually by the clinical services. Ultrasonographic examination for gallstones was done after an overnight fast with the gallbladder in a distended state using a Philips Healthcare, Model iU22 instrument using a curvilinear transducer probe i.e. C5-1 (3–5-MHz frequency). *HBB* mutations were detected using the PCR-based amplification refractory mutation system (ARMS-PCR) in all cases [15]. Alpha (α-)-globin gene deletions were tested in 76 patients by a single-tube multiplexed GAP-PCR assay for eight common deletions, viz., $-\alpha^{3.7}$ (rightward) and $-\alpha^{4.2}$ (leftward), $--^{SEA}$ (Southeast Asian), $--^{MED}$ (Mediterranean), $--^{SA}$ (South African), $--^{THAI}$ (Thailand), $--^{FIL}$ (Filipino), and $-(\alpha)^{20.5}$ [16]. Single-tube multiplex-PCR assay was used to screen for the presence of $\alpha\alpha\alpha^{anti\ 3.7}$ and $\alpha\alpha\alpha^{anti\ 4.2}$ triplications in 76 patients. The Xmn-1$^{G}\gamma$ polymorphism at position -158 of the *HBG2* promoter (rs7482144) was studied by PCR-RFLP in 76 patients [17].

Statistical analysis

Discrete categorical data were presented as *n* (%) and continuous data as mean ± S.D. and range or median

and interquartile range, as appropriate. The normality of quantitative data was checked by measures of Kolmogorov-Smirnov tests. Our data was skewed and thus, comparisons for two groups (non-GS/GS) were made by the Mann-Whitney test. For more than two groups, the Kruskal-Wallis test followed by the Mann-Whitney test for relevant subsets was applied. Proportions were compared using χ^2 or Fisher's exact tests, as applicable. All statistical tests were two-sided and performed at a significance level of $\alpha = 0.05$. Analyses were conducted using IBM SPSS STATISTICS software (version 22.0).

Results

Demographic and clinical data

The mean age of the patients was 26.2 ± 5.3 years (range 13–43 years, median 26 years), Table 1. Sixty-seven patients (65.7%) were males. Chronic hypertransfusion therapy had been initiated in all except 3 before the age of 5 years. All patients, with the exception of one, required at least one transfusion per month. Twenty-three patients (22.5%) had previously undergone splenectomy. Of the remainder, 49 (48.0%) showed variable degrees of splenomegaly. Fifty-eight patients (56.9%) had hepatomegaly.

Liver function tests

Mean total bilirubin was 2.35 ± 1.61 mg/dL (range 0.51–9.2) while unconjugated bilirubin was 1.77 ± 1.30 mg/dL (range 0.37–7.76). The total bilirubin level was in a normal range (reference range for laboratory < 1.0 mg/dL) in only 19/102 patients (18.6%) and was > 5.0 mg/dL in 7 patients (6.9%). Likewise, unconjugated bilirubin was normal (reference range for laboratory < 0.7 mg/dL) in only 16 patients (15.7%). AST, ALT, ALP, and albumin levels were 40.98 ± 33.11 mg/dL (range 15.46–288), 44.99 ± 34.86 (7.67–219), 105.71 ± 38.62 (46.54–222), and 4.45 ± 0.37 (2.66–5.19), respectively. Overall, 53 (52%) of the patients showed elevation of at least one of the 3 liver enzymes.

Table 1 Distributions of clinical and laboratory parameters between the *UGT1A1* promoter genotypes

Parameter	(TA)7/7 (*n* = 18)	(TA)6/7 *n* = 48)	(TA)6/6 (*n* = 36)	*p* value (Kruskal-Wallis test)
Age in years (median, range)	24.5 (13–32)	25.5 (18–43)	27 (19–35)	0.303
Age at initial diagnosis, months (median, range)	6.5 (1–60)	8 (1–72)	6 (2.5–96)	0.668
Age at first transfusion, months (median, range)	8.5 (1–60)	10 (3–72)	8.5 (3–96)	0.384
Splenectomized, *n* (%)	3 (16.7)	8 (16.7)	12 (33.3)	0.156
Splenomegaly, *n* (%)	9 (50)	22 (45.8)	18 (50)	0.914
Hepatomegaly, *n* (%)	12 (66.7)	28 (58.3)	18 (50)	0.487
Total bilirubin (mg/dL), mean ± S.D.; median (range)	4.26 ± 2.52; 4.14 (0.9–9.2)	2.02 ± 0.94; 1.89 (0.65–4.09)	1.84 ± 0.96; 1.62 (0.51–4.79)	< 0.001
Unconjugated bilirubin (mg/dL), mean ± SD; median (range)	3.24 ± 2.07; 3.54 (0.6–7.76)	1.5 ± 0.74; 1.38 (0.54–3.27)	1.4 ± 0.87; 1.15 (0.37–4.69)	0.002
AST, U/L (mean ± SD, range)	36.83 ± 24.53 (15.94–125.25)	40.1 ± 38.26 (19.58–288)	44.21 ± 29.74 (15.46–156.23)	0.354
ALT, U/L (mean ± SD, range)	39.58 ± 40.9 (8.0–184.79)	42.55 ± 31.66 (7.67–219)	50.97 ± 35.88 (11.56–188.53)	0.123
Alkaline phosphatase, U/L (mean ± SD, range)	115.53 ± 38.81 (57.86–193.91)	105.94 ± 39.58 (46.54–222)	100.51 ± 37.32 (50–212.6)	0.308
Albumin, g/dL (mean ± SD, range)	4.54 ± 0.3 (3.96–4.99)	4.45 ± 0.41 (2.66–5.05)	4.41 ± 0.35 (3.71–5.19)	0.389
HBSAg seropositive, *n* (%)	2 (11.8) (available in 17/18 pts)	5 (12.5) (available in 40/48 pts)	1 (2.85) (available in 35/36 pts)	-*
Anti-HCV seropositive, *n* (%)	1 (5.9) available (in 17/18 pts)	5 (12.5) (available in 40/48 pts)	4 (11.4) (available in 35/36 pts)	-*
HIV seropositive, *n* (%)	0 (0) (available in 17/18 pts)	0 (0) (available in 40/48 pts)	1 (2.85) (available in 35/36 pts)	-*
Gallstones on USG, *n* (%)	3 (16.7)	8 (16.7)	3 (8.3)	0.505 (χ^2 test)

ALT and AST alanine and aspartate aminotransferases, *HBSAg* hepatitis B surface antigen, *HCV* hepatitis C virus, *HIV* human immunodeficiency virus, *USG* ultrasonography

*Not analyzed statistically due to small numbers in each subgroup

Viral serology

Among the 92 patients in whom records of serological testing for viral markers were available, 19 (18.6%) were positive for at least 1 viral infection: 8 out of 92 (8.7%) were hepatitis B surface antigen positive, 10 out of 92 (10.9%) were reactive for anti-HCV antibodies, and 1 out of 92 was HIV-reactive (Table 1). None of these 19 patients had more than one viral infection. All were on standard management protocols, and on comparing the mean total as well as unconjugated bilirubin levels in the 18 patients who were positive for hepatitis B or C versus the non-reactive ones, there was no statistically significant difference in either of the values ($p = 0.135$ and 0.155 for total and unconjugated bilirubin respectively using the Kruskal-Wallis test).

Similarly, on comparing the mean AST, ALT, and ALP levels in patients positive for hepatitis B/C versus the mean levels in the non-reactive ones, there was no statistically significant difference in either of the values ($p = 0.694$, 0.289, and 0.285 for AST, ALT, and ALP respectively using the Kruskal-Wallis test).

β-Globin gene mutations

ARMS-PCR revealed the underlying *HBB* mutations in all the 102 cases (representing 204 β-globin alleles). Various mutations detected and their frequencies in 204 alleles from 102 patients are listed in Table 2.

α-Globin gene deletions and triplications

On performing multiplex GAP-PCR in 76 patients, the $-\alpha^{3.7}$ deletion was detected in 11 (14.5%) while the $-\alpha^{4.2}$ deletion was found in two patients (2.6%). Other α-globin gene deletions or triplications were not detected in any of the 76 patients tested. Total and unconjugated bilirubin did not differ significantly ($p = 0.366$ and 0.190 respectively) in 13 patients with an α-globin gene deletion versus those without them.

Table 2 Distribution of *HBB* mutations within the study group (204 alleles)

Mutation	Type	No. of alleles (%)
IVS 1 position 5 (G>C)	β^o	53 (26.0)
619 bp deletion	β^o	49 (24.3)
IVS 1 position 1 (G>T)	β^o	31 (15.3)
Fr 8/9 (+G)	β^o	27 (13.4)
Fr 41/42 (-TCTT)	β^o	18 (8.9)
Cd 16 (-C)	β^o	9 (4.5)
Cd 15 (G>A)	β^o	4 (1.9)
-88 (C>T)	β^{++}	4 (1.9)
Cap +1 (A>C)	β^+	3 (1.5)
Fr 47/48 (+ATCT)	β^o	3 (1.5)
Cd 5 (-CT)	β^o	3 (1.5)
Total	–	204

Xmn-1 Gγ polymorphism

Among the 76 patients tested, 53 (69.7%) did not show the presence of the polymorphic site (−/− genotype), three (4.0%) were homozygous (+/+ genotype), and the remaining 20 (26.3%) were heterozygous (+/− genotype) for this polymorphism. Total and unconjugated bilirubin levels were not significantly different between the different Xmn-1 genotypes ($p = 0.157$ and 0.081 respectively).

UGT1A1 gene promoter/Gilbert syndrome

DNA sequencing revealed the Gilbert genotype i.e. homozygous (TA)7 at the *UGT1A1* gene promoter in 18/102 patients (17.7%), heterozygous (TA)6/7 genotype in 48 patients (47.1%), and wild-type (TA)6/6 genotype in 36 patients (35.3%) (Table 1). None of the patients showed a trinucleotide CAT insertion at nt.-85 to nt.-83 previously described in eastern Indians [18].

The mean total bilirubin in the 18 GS patients was 4.26 ± 2.52 mg/dL (range 0.9–9.2) and unconjugated bilirubin was 3.24 ± 2.07 mg/dL (range 0.6–7.76). The mean total and unconjugated bilirubin levels in the 48 heterozygous (TA)6/7 genotype patients were 2.02 ± 0.94 mg/dL (range 0.65–4.09) and 1.5 ± 0.74 mg/dL (range 0.54–3.27) respectively. Patients with homozygous wild-type (TA)6/6 genotype ($n = 36$) had total and unconjugated bilirubin levels of 1.84 ± 0.96 mg/dL (range 0.51–4.79) and 1.4 ± 0.87 mg/dL (range 0.37–4.69%) respectively. The mean total and unconjugated bilirubin in the three *UGT1A1* genotypes were statistically significantly different ($p < 0.001$ and $p = 0.002$ for total and unconjugated bilirubin respectively), as summarized in Table 1.

Cholelithiasis

Ultrasonography revealed gallstones in 14 patients (13.7%), while they were absent in 88 (86.3%). None of the 23 splenectomized patients had had a concomitant cholecystectomy. Patients with gallstones were older, with a mean age of 28.2 ± 6.4 years (range 17–39) vis-à-vis those without gallstones (25.8 ± 5.1 years, range 13–43). There was no statistically significant difference in total or unconjugated bilirubin levels in patients with and without gallstones with *p* values of 0.508 and 0.406 respectively (Table 3).

In addition, there was no statistically significant difference between the proportions of patients with gallstones among the three *UGT1A1* genotypes (Table 1). Of patients with gallstones, 3/14 (21.4%) were (TA)7/7, 8/14 (57.2%) were

Table 3 Bilirubin levels in patients with and without gallstones

Gallstones	No. of pts. (%)	Total bilirubin, mg/dL; mean ± SD (range)*	Unconjugated bilirubin, mg/dL; mean ± SD (range)#
Present	14 (13.7)	3.01 ± 2.62 (0.75–9.20)	2.22 ± 1.89 (0.60–7.76)
Absent	88 (86.3)	2.25 ± 1.38 (0.51–7.44)	1.70 ± 1.19 (0.37–6.80)

*$p = 0.508$ and # $p = 0.406$ (Mann-Whitney test)

(TA)6/7, and 3/14 (21.4%) were (TA)6/6 on genotyping, indicating that GS patients did not show a significantly higher frequency of gallstones.

At the end of the study, of the 83 TDβT patients with hyperbilirubinemia, 18 had the Gilbert genotype and 12 had one of the hepatotropic viral illnesses. In the remaining 53 cases, no direct cause of unconjugated hyperbilirubinemia could be detected by our testing. However, 30 of these patients had derangement at least one of the liver enzymes tested and 16 showed heterozygosity for *UGT1A1* polymorphism (TA)6/7. The former could indicate sub-clinical hepatic injury due to drugs/other viral infections/iron overload/alcohol while the latter may be contributing to the unconjugated hyperbilirubinemia in conjunction with other undetected factors.

Discussion

This prospective study exploring the relationship of GS with unconjugated hyperbilirubinemia and cholelithiasis in Indian TDβT patients is currently the largest such south Asian report for this patient group and the first from this region to assess the frequency of gallstones in relation to the Gilbert genotype in these patients.

High frequency of Gilbert genotype Since the genomic locations of *UGT1A1* (chr. 2q37.1) and *HBB* (chr. 11p15.4) are unlinked, our β-thalassemic patients' high frequency of the homozygous (TA)7 promoter (17.7%) likely represents GS' high frequency in the north Indian population. GS may well represent the commonest genetic syndrome known in humans [19]. Among previous studies from India that studied this rs3064744, comparisons with Chiddarwar et al. and Aggarwal et al. (with GS frequencies of 23.5% and 15.6% respectively) may be inappropriate as they recruited patients with hyperbilirubinemia [20, 21]. On the other hand, Premawardhana et al. evaluated 1617 subjects from 24 countries/regions and found the highest worldwide frequencies in India, Sri Lanka, and Bangladesh (19.2–24.0%) [22]. Chaouch et al. reported that 21% of their Tunisian subjects with β-thalassemia trait had at least 7 (TA) repeats on each of their *UGT1A1* alleles [23]. The prevalence of GS is variable in populations outside south Asia, being 5–15% among Europeans, 6–18% among Africans, 1–5% in south-east Asia, ~12% in South America, and only 0–2.5% among Pacific islanders [22, 24].

Influence of GS on bilirubin and liver enzyme levels in TDβT Correlations between the number of (TA) repeats inherited with unconjugated and total bilirubin levels are well established. Results similar to ours were reported by Dabke et al. where the mean total bilirubin of (TA)7/7, 6/7, and 6/6 groups were 2.95, 2.10, and 1.28 mg/dL respectively [11]. Galanello et al. showed that the mean unconjugated bilirubin was 3.5 mg/dL in the 26 Gilbert genotype cases and 1.0 mg/dL in 133 (TA)6/6 TDβT patients [12]. Tzetis et al. reported that 16 (TA)7/7 patients had mean total bilirubin levels of 3.6 mg/dL, 50 (TA)6/7 had 1.8 mg/dL, and 42 (TA)6/6 had 1.3 mg/dL [13]. Previous studies examining GS in thalassemia syndromes are summarized in Table 4.

Gallstones in TDβT and GS Ultrasonographic examination revealed gallstones in 14 of our patients (13.7%) with no statistically significant differences in total or unconjugated bilirubin levels in these patients versus the rest. On the other hand, Chaouch et al. detected cholelithiasis in 50 of 76 β-thalassemia minor patients and 47 of 75 sickle cell anemia patients in Tunisia. Unconjugated bilirubin levels were significantly higher in patients with gallstones than those without gallstones [23]. Explanations for our discordant findings may include that bilirubin is an actively metabolized chemical moiety with levels being affected by recent transfusions, viral infections, and background genetic factors as well as differences in conventional risk factors for gallstones like diet, obesity, recent weight loss, hormonal influences, and alcohol use [3, 8, 9, 20, 25].

We also found no statistically significant differences in the proportions of patients with gallstones within each of the three *UGT1A1* genotypes. This surprising finding is replicated only rarely in prior studies: an Israeli paper also did not find GS to correlate with cholelithiasis in their 323 adult patients with Gaucher disease [26]. The more frequent finding has been akin to that of Origa et al. who reported the frequency of gallstones in 858 TDβT patients to be significantly higher (41.7%) among (TA)7/7 vis-à-vis (TA)6/7 (30.5%) and (TA)6/6 (26.2%) genotypes [3]. The mean age when gallstones were detected was significantly lower by 4 years in

Table 4 Results of salient prior studies on the Gilbert syndrome in TDβT patients compared with our findings

S No.	Lead author (ref), year, *country*	No. of TDβT patients; age range/ distribution	% TDβT cases with (TA)7/7, bilirubin levels and gallstones in them	% with (TA)6/7, bilirubin levels and gallstones	% with (TA)6/6, bilirubin levels and gallstones	Status of viral infections	Status of liver enzymes
1	Dabke [11], 2014, *India*	$n = 40$; not provided	12.5%, mean total bilirubin 2.95 ± 0.63 mg/dL; gallstones not studied.	32.5%, mean total bilirubin 2.1 ± 1.65 mg/dL; gallstones not studied.	50%, mean total bilirubin 1.28 ± 0.07 mg/dL; gallstones not studied.	N/A	–
2	Galanello [6], 2001, *Italy*	$n = 261$; 6 to 30 years	10%, mean unconjugated bilirubin 3.5 ± 2.4 mg/dL; 42.3% had gallstones	39.1%. No data on bilirubin or gallstones in this group	50.1%, mean unconjugated bilirubin 1.0 ± 0.5 mg/dL; 18% had gallstones.	N/A	No significant difference in ALT among these genotypes
3	Tzetis [13], 2001, *Greece*	$n = 108$; 4 to 34 years	15%, mean total bilirubin 3.6 ± 2.2 mg/dL; gallstones not studied.	46%, mean total bilirubin 1.8 ± 1.2 mg/dL; gallstones not studied.	39%, mean total bilirubin 1.3 ± 0.6 mg/dL; gallstones not studied.	N/A	–
4	Al Fadhli [5], 2013, *Kuwait*	$n = 70$; mean 28 ± 8.4 years	5%, mean total bilirubin 4.2 ± 2.5 mg/dL; gallstones data on TDβT N/A	63%, mean total bilirubin 1.7 ± 1.0 mg/dL. Gallstone data on TDβT N/A	27%, mean total bilirubin 1.2 ± 0.5 mg/dL; gallstone data on TDβT N/A	N/A	–
5	Origa [3], 2008, *Italy*	$n = 858$; 1 to 61	10%, data on bilirubin in this genotype N/A; 41.7% had gallstones	30.5%, data on bilirubin in this genotype N/A; 30.5% had gallstones	26.2%, data on bilirubin in this genotype N/A; 26.2% had gallstones	70% were HCV + ve; significantly more frequent gallstones in this group.	–
6	Wing Yan Au [25], 2003, *China*	$n = 94$; median age: 20 years	19.6% with (TA)7/7 and 26.8% with Gly71Arg. Bilirubin and gallstone status not recorded but mentioned as significantly increased.	N/A	N/A	N/A	–
7	Present study, 2020, *India*	$n = 102$; 13 to 43 years	17.65%, mean unconjugated bilirubin 3.24 ± 2.07 mg/dL. 16.7% had gallstones.	47.05%, mean unconjugated bilirubin 1.5 ± 0.74 mg/dL; 16.7% had gallstones.	35.3%, mean unconjugated bilirubin 1.4 ± 0.87 mg/dL; 8.3% had gallstones.	10.9% were HCV reactive & 8.7% were HBsAg reactive.	No significant difference in liver enzyme among these genotypes

ALT serum alanine transferase; *HBSAg* hepatitis B surface antigen; *HCV* hepatitis C virus infection; *N/A* data not reported; *TDβT* transfusion-dependent β-thalassemia

their (TA)7/7 patients compared with the rest. However, 70% of their study population were HCV-positive, which is known to influence the incidence of gallstones [27]. In contrast, only one of our 12 patients with gallstones in whom viral serology reports were available were positive for anti-HCV antibodies and HBSAg. In our study too, the mean age of patients with gallstones was higher i.e. 28.2 ± 6.4 years (range 17–39 years) while the age of those without gallstones was 25.8 ± 5.1 years (range 13–43 years).

Galanello et al. reported that 42.3% of their 26 TDβT patients with the (TA)7/7 genotype had cholelithiasis or a previous cholecystectomy compared with 18.0% of the 133 patients with (TA)6/6 arrangement [6]. The only previous Indian study on GS in TDβT did not report the status of gallstones in their patients [11].

Transfusion-transmitted infections in TDβT Nagral et al. recently reported the pre-treatment bilirubin levels in their cohort of 120 HCV-positive TDβT patients to be only mildly elevated at 1.7 ± 0.8 mg/dL [28]. They did not compare these with HCV-negative patients or correlate with the presence of GS. We did not detect significant differences in AST, ALT, and ALP levels in hepatitis B/C-positive patients versus non-reactive ones. Ragab et al. studied 100 Lebanese TDβT patients among which 64 were HCV-reactive and 36 were HCV-non-reactive [29]. No significant difference was seen in the ALT, AST, and ferritin levels among these groups. However, Wahidiyat et al. found 5 out of 621 subjects to be positive for HBsAg (0.8%), 111 positive for anti-HCV (17.8%), and 5 positive for both HBsAg and anti-HCV (0.8%). Subjects positive for hepatitis B, C, or both showed significantly higher values of AST, ALT, and ferritin compared with their negative counterparts [30]. AST and ALT values, denoting hepatocellular injury, are raised predominantly in acute phases of the infections. In our viral hepatitis patients, although the mean serum AST and ALT were higher than in those who did not have the infections, these differences did not reach statistical significance. Enzyme levels typically decline if the infection is either controlled naturally or through therapy and are often normal in chronic patients and even those displaying varying degrees of histological damage in the liver including cirrhosis [31].

Limitations of this study Although this is the largest such study from India, our sample size was much smaller than that of Origa et al. [3] and slightly smaller than Galanello et al. [6, 12] and Tzetis et al. [13]. Recruiting more patients might have helped clarify the relationship between gallstones and GS further. It may have also revealed the occasional patients with the CAT trinucleotide insertion. Testing for genetic polymorphisms in *UGT1A1*'s coding regions (and not just the promoter motif) or other loci affecting bilirubin levels was not done. These may be best accomplished by multigene panels using next-generation sequencing.

Conclusion

The Gilbert genotype's high frequency and significant association with hyperbilirubinemia suggest that *UGT1A1* promoter sequencing should be considered in TDβT patients whose jaundice remains unexplained after treatable causes like viral infections, chelator toxicity, or transfusion-related hemolytic reactions are excluded. The low cost and widespread availability of Sanger sequencing likely make it economically more feasible than repeated biochemical and virological testing and may assuage patient anxiety. Future larger studies could focus on the analysis of other *UGT1A1* gene defects that may very rarely cause GS or polymorphisms in other genes associated with increased bilirubin levels to further refine genetic prediction of hyperbilirubinemia in these patients.

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Code availability Not applicable.

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Data availability All data mentioned in this paper are available with the corresponding author and will be provided upon request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval This research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013). It was approved by the authors' Institutional Review Board.

Consent to participate Informed consent was obtained from all study participants.

Consent for publication Consent was obtained from all individuals included in this study to publish their scientific data in an aggregated anonymized manner.

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