

Incidence and Risk of Gallstone Disease in Gilbert's Syndrome Patients in Indian Population



Govardhan Bale^{*}, Urmila S. Avanthi^{*}, Padaki N.Rao[†], Mithun Sharma[†], Duvvur N.Reddy[†], Vishnubhotla V.Ravi Kanth^{*}

^{*}Asian Healthcare Foundation, 6-3-661, Somajiguda, Hyderabad 500082, Telangana, India and [†]Asian Institute of Gastroenterology, 6-3-661, Somajiguda, Hyderabad 500082, Telangana, India

Background/objectives: Individuals with Gilbert's syndrome (GS) harbor mutations in the *UGT1A1* gene and are known to have elevated levels of bilirubin, which enhances the risk for gall stone formation. The aim of this study is to screen Indian patients with GS for the incidence of gall stone disease. **Methods:** Individuals with persistently elevated serum bilirubin levels were genotyped for two polymorphisms (rs8175347; rs4148323) in *UGT1A1* gene to confirm GS in them. Flanking regions of the above polymorphisms were amplified followed by direct sequencing. Ultrasonography was done to detect gallstone disease. Clinical data, including assessment of liver function, circulating levels of total and direct bilirubin, as well as routine hematological parameters were obtained as per standard procedures (Autoanalyzer). **Results:** Of the total 1621 individuals subjected to genotyping, 1191 (1149 males of 29.6 ± 11.3 years with mean BMI of 22.1 ± 3.7 kg/m² and 42 females of 30.8 ± 14.8 years with mean BMI of 20.9 ± 3.7 kg/m²) were confirmed to have GS. Gall bladder abnormalities including cholelithiasis ($n = 106/1191$; 8.9%), polyps ($n = 18/1191$; 1.5%) and gallbladder wall thickening ($n = 17/1191$; 1.4%) were noted. Incidence of gall stone disease was observed in 103 males (out of 1149) and 3 females (out of 42) indicating the risk of the disease to be 9.0% and 7.1% respectively in males and females with GS. **Conclusion:** Early recognition of GS by genetic analysis is required before these patients with intermittent episodes of jaundice run the risk of unnecessary operations on their bile ducts from the mistaken assumption ascribing the jaundice to a stone which has been left behind. (J CLIN EXP HEPATOL 2018;8:362–366)

Gallstone disease (GD) is a common gastrointestinal ailment causing substantial burden to the health care system with 10–20% of the patients developing symptoms.¹ While genetic predisposition and environmental factors have a profound influence on disease causation,² demographic and environmental risk factors³ (including age, sex and body mass index,) are relevant in this regard. The prevalence of Gilbert's syndrome is 6% in South Indian ethnicity⁴ and it affects 7.4% of adult population in North India,⁵ where in cholesterol

stones, composed principally of cholesterol monohydrate crystals are encountered as opposed to pigment stones (composed of calcium bilirubinate) that are encountered in South India.^{5,6}

Pigment gallstones are formed due to excess secretion of bilirubin,⁷ arising from hemolysis, ineffective erythropoiesis, or pathologic enterohepatic cycling of unconjugated bilirubin, into the bile. An augmented enterohepatic cycling of bilirubin emanating from increased absorption of unconjugated bilirubin has been proposed as the cause of pigment gallstones.⁸ Pigment gallstones are classified descriptively as "black" or "brown", and are mostly composed of calcium hydrogen bilirubinate, which is polymerized and oxidized in "black" stones but remains unpolymerized in "brown" stones. Bilirubin monoconjugates that are increased in several conditions, such as in Gilbert's syndrome and chronic hemolysis, may play a pivotal role in pigment stone formation where the unconjugated monohydrogenated bilirubin co-precipitates with calcium. Generally, pigment stones are formed by the precipitation of bilirubin in bile, with black stones associated with chronic hemolytic states, and Gilbert's syndrome.⁹

Gilbert's syndrome (GS) is a genetic disorder that results from increased serum unconjugated bilirubin levels in the absence of liver disease or overt hemolysis.¹⁰ Raised bilirubin alone can be a manifestation of more

Keywords: Gilbert's syndrome, gallstone disease, genetic polymorphisms, unconjugated bilirubin, *UGT1A1* gene

Received: 18 October 2017; Accepted: 24 December 2017; Available online: 30 December 2017

Address for correspondence: Vishnubhotla Venkata Ravi Kanth, Asian Healthcare Foundation, 6-3-661, Somajiguda, Hyderabad 500 082, India. Tel.: +91 40 23378888; fax: +91 40 23324255.

E-mail: ravikanth.aig@gmail.com

Abbreviations: ALT: alanine transaminase; AST: aspartate amino transferase; BMI: body mass index; DNA: deoxyribose nucleic acid; ERCP: Endoscopic Retrograde Cholangio Pancreatography; EUS: endoscopic ultrasonogram; GD: gall stone disease; GS: Gilbert's syndrome; GWAS: genome wide association disease; MRCP: Magnetic Resonance Cholangio Pancreatography; PCR: polymerase chain reaction; SNPs: single nucleotide polymorphisms; *UGT1A1*: UDP glucuronosyltransferase family 1 member A1

<https://doi.org/10.1016/j.jceh.2017.12.006>

serious disease.¹¹ Various tests have been proposed in an attempt to provide a firm diagnosis of Gilbert's syndrome. Two polymorphisms in *UGT1A1* namely G71R in exon 1 (rs4148323) and A(TA)₇TAA in the TATA box (rs8175347) of the promoter region are the most prevalent and are responsible for GS.^{12,13} A simple dinucleotide repeat polymorphism (TA)₅₋₈, in the TATA box of the *UGT1A1* gene is associated with reduced expression of the hepatic enzyme and results in the chronic unconjugated hyperbilirubinemia that occurs in Gilbert syndrome.^{14,15}

A polymorphism (rs8175347) in *UGT1A1* gene in homozygous state is associated with GS and unconjugated hyperbilirubinemia.¹⁶ It is revealed that the length of TA repeats in the promoter region is an important modulator of enzyme activity; the greater the number of repeats higher the serum bilirubin levels and cholelithiasis risk.^{16,17} Furthermore, genome-wide association studies (GWAS) of three large Caucasian cohorts have identified single nucleotide polymorphisms (SNPs) in *UGT1A1* gene that are responsible for 18% of clinical variability of serum bilirubin.¹⁸ Cholelithiasis risk is partially mediated by genes impacting bilirubin metabolism,^{19,20} and earlier studies from our lab have identified the association of a variant in *UGT1A1* gene with pigment gallstone disease in South Indian population.²¹ Previous studies reported coexistence of gallstones with Gilbert's Syndrome.²²⁻²⁵ In the present study we report the coexistence of gallstone disease in Indian patients affected with Gilbert syndrome.

METHODS

Study Population

A total of 1621 individuals (1506 men and 115 women), with high bilirubin levels, who visited the hepatology clinics between September 2009 and April 2017, were enrolled into the study. Anthropometric data were obtained from these individuals and blood samples (2 ml) were collected from the patients for biochemical and genetic analyses. The study was approved in institutional ethics committee.

Genotyping

Gilbert's syndrome in the patients was confirmed by genetic screening of variants in *UGT1A1* gene. Genomic DNA was isolated from peripheral blood leukocytes of whole blood by commercial kit (Bioserve Biotechnologies, India) following manufacturer's instructions. DNA concentration and purity was measured with NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and agarose gel electrophoresis respectively. Variants namely *UGT1A1* (TA)_n (rs8175347) and G7R(G>A) (rs4148323) in *UGT1A1* gene were genotyped in all patients by polymerase chain reaction (PCR) followed by direct DNA sequencing. Briefly, 100 ng of DNA along with 10 pmol/ μ l primers (Forward primer F-5' TCCCTGCTACCTTTGTGGAC3'

and Reverse primer R-5' CTGGGTAGCCTCA AATCCA3'), dNTPs (10 mM each) and Taq polymerase (5 U/ μ l) to generate a 700-bp fragment. The PCR reactions conditions were initial denaturation at 95 °C for 5 min; followed by 35 cycles at 95 °C for 60 s, 60 °C for 60 s, and 72 °C for 60 s and by a final extension step at 72 °C for 7-min. The amplicons were purified using AMPure magnetic beads (Agencourt AMPure XP-Beckman Coulter) and dye Termination cycling Sequencing was done by sequencing ready reaction Kit (Beckman) and was directly sequenced by Beckman coulter (Genomelab GeXp). Sequence analysis was performed using software (Beckman Coulter CEQTM 8000, Fullerton, CA 92834-3100).

Biochemical Analyses

Total and direct bilirubin levels, alanine transaminase (ALT), Aspartate amino transferase (AST) and other liver function parameters were estimated in serum using commercially available kits (Randox laboratories, Crumlin, Antrim, UK). Hemoglobin level, reticulocyte count and other hemogram parameters were estimated by auto analyzer (Beckman coulter LX750, Miami, FL).

RESULTS

Clinical Characteristics

Of the 1621 individuals referred for genetic testing for Gilbert's syndrome, 1157 were of South Indian (SI) and 464 were of East Indian (EI) ethnicity with a BMI (mean \pm SD) of 22.04 \pm 3.8 and 22.01 \pm 3.2 respectively. The ultrasound investigations were conducted for 1240 individuals in whom Gilbert's syndrome could be confirmed in 1191 individuals including 1149 males (96.5% mean age 29.6 \pm 11.3 years; mean BMI 22.1 \pm 3.7 kg/m²) and 42 females (3.5% mean age 30.8 \pm 14.8 years; mean BMI 20.9 \pm 3.7 kg/m²). Coexistence of GS with gallbladder abnormality was noted in greater number of males (137/141; 97.2%) than in females. Demographic and clinical characteristics between Gilbert's syndrome with and without gallstone disease (GS-GD) for 1191 patients are as presented in Table 1. Even though no significant difference was observed, total bilirubin levels were high and indirect bilirubin was higher than the direct bilirubin in both the groups. Related parameters indicative of liver function as well as hematological parameters did not reveal any significant changes as shown in Table 1. It was also interesting to note that there was no significant difference in the BMI between the groups.

UGT1A1 Gene Polymorphism and Gilbert's Syndrome

In a total of 1240 individuals suspected for Gilbert's syndrome, 58 subjects were wild, 258 were heterozygous and 924 were homozygous for the rs8175347 dinucleotide

Table 1 Demographic and Clinical Characteristics.

	GS with GD Mean ± SD	GS Mean ± SD	Ref. Range
Age	34.3 ± 13.6	29.1 ± 11.2	
Gender n(M/F)	103/3	1012/38	
BMI	20.75 ± 2.95	22.04 ± 3.72	
Total Bilirubin (mmol/L)	3.67 ± 1.92	3.8 ± 1.8	0.3–1.2
Direct Bilirubin (mmol/L)	0.70 ± 0.63	0.9 ± 0.4	Up to 0.2
Indirect Bilirubin (mmol/L)	2.97 ± 1.68	2.9 ± 1.7	
ALT (U/L)	33.97 ± 18.08	31.6 ± 19.3	0–40
AST (U/L)	30.17 ± 10.49	29.5 ± 16.4	Up to 40
ALP (U/L)	82.91 ± 27.97	84.2 ± 28.7	30–120
Total protein (mmol/L)	7.35 ± 0.58	7.6 ± 0.4*	6–8
Albumin (mmol/L)	4.29 ± 0.67	4.6 ± 0.3*	3.5–5.2
Globulin (mmol/L)	3.01 ± 0.42	3.0 ± 0.3	2.3–3.5
Total WBC (cell/mm ³)	7807.61 ± 2831.04	7679.4 ± 2467.1	4000–10000
RBC (millions/mm ³)	4.50 ± 0.93	4.9 ± 1.0*	3.8–4.8
Hemoglobin (mmol/L)	13.51 ± 2.46	14.6 ± 2.6*	12.0–15.0
HCT (%)	39.24 ± 6.65	41.7 ± 8.5*	36–46
Platelets (×10 ⁶ /mm ³)	1.93 ± 0.76	2.4 ± 0.7*	1.5–4.1

GS, Gilbert's syndrome; GD, gallstone disease; Ref, reference; BMI, body mass index; ALT, alanine transaminase; AST, aspartate amino transferase; ALP, alkaline phosphatase; WBC, white blood cells; RBC, red blood cells; HCT, hematocrit.

* $P > 0.05$.

repeat polymorphism (TA)₅₋₈. For the rs4148323 single nucleotide polymorphism 1102 subjects were wild, 129 were heterozygous and 9 were homozygous. Of these, 129 were compound heterozygous for both the variants. Gilbert's syndrome was confirmed in 1191 of the 1240 subjects (96%) based on the genotype.

Gilbert's Syndrome and Gall Stone Disease

A total of 1191 subjects were positive for Gilbert's syndrome, of these 11.8% ($n = 141$) had gall bladder abnormalities including cholelithiasis ($n = 106/1191$; 8.9%), polyps ($n = 18/1191$; 1.5%) and gallbladder wall thickening ($n = 17/1191$; 1.4%) respectively (Figure 1). In the present analysis, it was seen that 9.0% of males ($n = 103/1149$) and 7.1% of females ($n = 3/42$) were at risk for gallstone disease.

DISCUSSION

Gilbert's syndrome is associated with mildly elevated levels of unconjugated bilirubin in absence of liver disease and is characterized by episodes of mild, intermittent jaundice. It is well known that a polymorphism in the promoter region of *UGT1A1* gene is responsible for the elevated unconjugated hyperbilirubinaemia leading to GS,²⁶ which is usually diagnosed incidentally during a lab investigation or when a light yellowish tinge of conjunctivae is

noticed; both the features (elevated bilirubin levels and yellow eyes) tend to be repetitive causing undue anxiety in the patients and their attendants. Individuals with

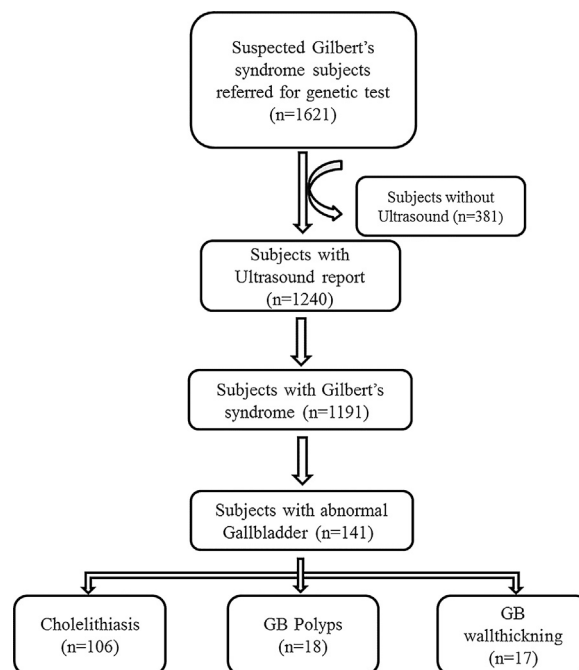


Figure 1 Samples consort diagram.

Gilbert's syndrome also develop gallstones necessitating cholecystectomy. It is not uncommon in clinical practice that the intermittent episodes of jaundice are ascribed to gall stone(s) left behind, leading to the risk of unwarranted operations on their bile ducts. Thus, it is prudent to determine the coexistence of Gilbert's syndrome in individuals with gallstone disease. In absence of detailed knowledge in the Indian population in this regard, we conducted the present study to determine how frequently these conditions coexist.

The present study involved individuals with increased levels of circulating bilirubin and comparisons were made between patients confirmed for Gilbert's syndrome with or without concomitant gall stone disease in them. Bilirubin biosynthesis starts from hemoglobin metabolism, where heme is metabolized into biliverdin and then is converted to bilirubin which is termed as unconjugated or indirect bilirubin. The unconjugated bilirubin bound with albumin is transported via the bloodstream to the liver where it undergoes glucuronidation into conjugated bilirubin by the UDP-glucuronosyltransferase 1(UGT family enzyme); only UGT1A1 is involved in bilirubin conjugation.²⁷ In our results we found lower conjugated bilirubin and higher unconjugated bilirubin levels but it's not statistically significant and there was significantly lower albumin and total proteins in GS subjects between with GD and without GD; albumin and UGT family of enzymes play an important role in conversion of unconjugated bilirubin into conjugated bilirubin. An increased unconjugated bilirubin has been proposed as the cause of pigment gallstones.⁸ We also found significantly lower hemoglobin, RBC and hematocrit which were haemolytic cause for the hyperbilirubinaemia.

Results obtained in the present study indicated certain interesting findings about the incidence of gallstone disease in males and females. It is well known that middle aged, obese females also tend to develop gallstone disease. The obtained results (Table 1) indicated higher prevalence of Gilbert's syndrome and gallstone disease in males, as opposed to females. While the increased tendency of females to develop gallstone disease is well recognized in Northern India.²⁸ It is known that female gender,²⁹ obesity³⁰ are risk factors for gallstone disease but majority of the subjects were males in the present study. GS is predominantly a disease of males^{22,31} and in this study all subjects had normal BMI; which shows that GS independently of these parameters is associated with gallstone disease. Gilbert's syndrome subjects have been found to have gallstones ~5% in previous reports²⁵ and in the present study we found that 8.9% of Gilbert's syndrome (UGT1A1(TA)n promoter polymorphism) patients had gallstones.

Some of these subjects (with undiagnosed Gilbert's syndrome) undergo unnecessary investigations like MRCP (Magnetic Resonance Cholangio Pancreatography), ERCP

(Endoscopic Retrograde Cholangio Pancreatography) or EUS (Endoscopic Ultrasonogram) when "jaundice" is found to be associated with symptomatic or asymptomatic gallstones. In few subjects, elevated levels of bilirubin may appear during follow-up after laparoscopic cholecystectomy has been done, resulting again in needless investigations for a suspected retained common bile duct stone. Similar situation can arise in patients who have recovered after complete resolution from an episode of acute hepatitis when bilirubin is found to be "still elevated" during follow-up. Hence it is prudent to be aware of Gilbert's syndrome in any one with unconjugated hyperbilirubinaemia with normal hemoglobin levels associated with gallstone disease.

In conclusion, individuals with increased levels of circulating unconjugated bilirubin in gallstone disease are to be investigated for polymorphisms associated with Gilbert's syndrome.

CONFLICTS OF INTEREST

The authors have none to declare.

ACKNOWLEDGEMENTS

The authors acknowledge the individuals for consenting to participate in the study. We acknowledge the help rendered by Mrs. Ashwini Kaitha in genotyping the samples. Our sincere thanks are due to Professor C. Subramanyam and Dr. M. Sasikala for critical review and suggestions on the manuscript.

REFERENCES

1. Friedman GD, Raviola CA, Fireman B. Prognosis of gallstones with mild or no symptoms: 25 years of follow-up in a health maintenance organization. *J Clin Epidemiol.* 1989;42:127-136.
2. Lammert F, Sauerbruch T. Mechanisms of disease: the genetic epidemiology of gallbladder stones. *Nat Clin Pract Gastroenterol Hepatol.* 2005;2:423-433.
3. Miquel JF, Covarrubias C, Villaroel L, et al. Genetic epidemiology of cholesterol cholelithiasis among Chilean Hispanics, Amerindians, and Maoris. *Gastroenterology.* 1998;115:937-946.
4. Kulkarni RG, Lakshmi Devi KB, Ronghe V, Dinesh US. Gilbert's syndrome in healthy blood donors what next? *Asian J Transfus Sci.* 2016;10(January):63.
5. Tandon RK. Prevalence and type of biliary stones in India. *World J Gastroenterol.* 2000;6:4-5.
6. Jayanthi V, Palanivelu C, Prasanthi R, Methew S, Srinivasan V. Composition of gall stones in Coimbatore district of Tamil Nadu state. *Indian J Gastroenterol.* 1998;17:134-135.
7. Buch S, Schafmayer C, Volzke H, et al. Loci from a genome-wide analysis of bilirubin levels are associated with gallstone risk and composition. *Gastroenterology.* 2010;139:1942-1951.
8. Vitek L, Carey MC. Enterohepatic cycling of bilirubin as a cause of "black" pigment gallstones in adult life. *Eur J Clin Invest.* 2003;33:799-810.
9. Lambou-Gianoukos S, Heller SJ. Lithogenesis and bile metabolism. *Surg Clin North Am.* 2008;88:1175-1194.
10. Gilbert A, Lereboullet P. La cholémie simple familiale. *Semaine Med.* 1901;21:241-243.

11. Levine RA, Klatskin G. Unconjugated hyperbilirubinemia in the absence of overt hemolysis: importance of acquired disease as an etiologic factor in 366 adolescent and adult subjects. *Am J Med.* 1964;36:541–552.
12. Borlak J, Thum T, Landt O, Erb K, Hermann R. Molecular diagnosis of a familial nonhemolytic hyperbilirubinemia (Gilbert's syndrome) in healthy subjects. *Hepatology.* 2000;32:792–795.
13. Burchell B, Hume R. Molecular genetic basis of Gilbert's syndrome. *J Gastroenterol Hepatol.* 1999;14:960–966.
14. Bosma P, Chowdhury J, Bakker C, et al. The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *N Engl J Med.* 1995;333:1171–1175.
15. Monaghan G, Ryan M, Seddon R, Hume R, Burchell B. Genetic variation in bilirubin UDP-glucuronosyltransferase gene promoter and Gilbert's syndrome. *Lancet.* 1996;347:578–581.
16. Tsezou A, Thetis M, Giannatou E, et al. Gilbert syndrome as a predisposing factor for cholelithiasis risk in the Greek adult population. *Gen Test.* 2009;13:143–146.
17. Bosma PJ, Chowdhury JR, Bakker C, et al. The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *N Engl J Med.* 1995;333:1171–1175.
18. Benjamin EJ, Dupuis J, Larson MG, et al. Genome-wide association with select biomarker traits in the Framingham heart study. *BMC Med Genet.* 2007;8(suppl 1):S11.
19. Vogel A, Kneip S, Barut A, et al. Genetic link of hepatocellular carcinoma with polymorphisms of the UDP-glucuronosyltransferase UGT1A7 gene. *Gastroenterology.* 2001;121:1136–1144.
20. Vasavda N, Menzel S, Kondaveeti S, et al. The linear effects of alpha-thalassaemia, the UGT1A1 and HMOX1 polymorphisms on cholelithiasis in sickle cell disease. *Br J Haematol.* 2007;138:263–270.
21. Ravikanth VV, Rao GV, Govardhan B, et al. Polymorphisms in UGT1A1 gene predispose south Indians to pigmentous gallstones. *J Clin Exp Hepatol.* 2016;6:216–223.
22. Peel AL, Ritchie HD. Gilbert's syndrome in patients with gallbladder stones. *Ann R Coll Surg Engl.* 1974;55:184.
23. Milton JN, Sebastiani P, Solovieff N, et al. A genome-wide association study of total bilirubin and cholelithiasis risk in sickle cell anemia. *PLoS ONE.* 2012;27:e34741.
24. Foulk WT, Butt HR, Owen Jr CA, Whitcomb Jr FF, Mason HL. Constitutional hepatic dysfunction (Gilbert's disease): its natural history and related syndromes. *Medicine.* 1959;38:25–46.
25. Powell LW, Hemingway E, Billing BH. Idiopathic unconjugated hyperbilirubinemia (Gilbert's syndrome). A study of 42 families. *N Engl J Med.* 1967;277:1108–1112.
26. Krawczyk M, Wang DQ, Portincasa P, et al. Dissecting the genetic heterogeneity of gallbladder stone formation. *Semin Liver Dis.* 2011;31:157–172.
27. Perera MA, Innocenti F, Ratain MJ. Pharmacogenetic testing for uridine diphosphate glucuronosyltransferase 1A1 polymorphisms: are we there yet? *Pharmacotherapy.* 2008;28:755–768.
28. Unisa S, Jagannath P, Dhir V, Khandelwal C, Sarangi L, Roy TK. Population-based study to estimate prevalence and determine risk factors of gallbladder diseases in the rural Gangetic basin of North India. *HPB.* 2011;13:117–125.
29. Novacek G. Gender and gallstone disease. *WMW Wiener Medizinische Wochenschrift.* 2006;156:527–533.
30. Stampfer MJ, Maclure KM, Colditz GA, Manson JE, Willett WC. Risk of symptomatic gallstones in women with severe obesity. *Am J Clin Nutr.* 1992;55:652–658.
31. Kornberg A. Latent liver disease in persons recovered from catarrhal jaundice and in otherwise normal medical students as revealed by the bilirubin excretion test. *J Clin Invest.* 1942;21:299.