

## ORIGINAL ARTICLE

# Genetic testing of *UGT1A1* in the diagnosis of Gilbert syndrome: The discovery of seven novel variants in the Chinese population

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## Abstract

**Background:** Genetic testing of *UGT1A1* was used to facilitate the diagnosis of Gilbert syndrome, and analyze the distribution features of pathogenic variants in the Chinese population.

**Methods:** DNA was extracted from whole blood samples of patients with unconjugated hyperbilirubinemia, and sequencing of the *UGT1A1* gene was performed after PCR amplification. After alignment with reference sequences, the known pathogenic variants were identified, the variant spectrum was analyzed, and the pathogenicity of novel variants was predicted using online mutation prediction tools.

**Results:** A total of 117 patients were confirmed with Gilbert syndrome by *UGT1A1* genetic diagnosis, where the most common pathogenic variants included promoter A(TA)<sub>7</sub>TAA insertion and p.Gly71Arg missense variant. Following novel variants were also identified: p.Ala61Gly, p.Tyr67Phe, p.Leu166Alafs\*16, p.Arg240Lys, p.Ser306Phe, p.Arg341Gln, and p.Glu424\* variants.

**Conclusions:** Genetic testing of *UGT1A1* in clinical practices could facilitate confirming Gilbert syndrome and performing differential diagnosis. The pathogenic variant spectrum in the Chinese population was similar to other Asian populations. The novel pathogenic variants identified in this study require further investigation.

## KEYWORDS

Crigler–Najjar syndrome, genetic testing, Gilbert syndrome, hyperbilirubinemia, *UGT1A1* gene

## 1 | INTRODUCTION

*UGT1A1* gene (OMIM#191740), which is responsible for the coding of UDP-glucuronosyltransferase 1A1

(*UGT1A1*), contains five exons, while the promoter of the gene contains an A(TA)<sub>6</sub>TAA repetitive sequence that is closely associated with the initiation of gene transcription (Wang et al., 2020). *UGT1A1* gene is a member of the

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*UGT1A* subfamily, which shares four common exons from exon 2 to exon 5 with other members and has a unique exon 1. The *UGT1A* subfamily includes nine protein-coding genes encoding for functional proteins and four pseudogenes. *UGT1A1* is the major isoform responsible for the metabolism of many endogenous and exogenous compounds and is the most abundant one in the human liver. *UGT1A1* participates in the metabolism of endogenous unconjugated bilirubin and some exogenous drugs (such as irinotecan; Fretzayas et al., 2012; Gagne et al., 2002; Sanchez-Dominguez et al., 2018; Servedio et al., 2005). The pathogenic variant of the *UGT1A1* gene could lead to the reduction of enzymatic activities and consequently influence the level of unconjugated bilirubin, as well as the efficacies and adverse effects of relevant drugs. Hereditary hyperbilirubinemia that is mainly manifested by the simple elevation of unconjugated bilirubin includes Gilbert syndrome, and type I and II Crigler-Najjar syndrome, all of which are autosomal recessive hereditary disorders caused by *UGT1A1* gene defects (Steventon, 2020). Due to the compound heterozygous mutation or homozygous mutation, the *UGT1A1* enzyme activity in Gilbert syndrome patients could be reduced to about 30% of the normal level. The disease mainly occurs after adolescence, and the unconjugated bilirubin level is elevated to about 17–85  $\mu\text{mol/L}$ . Genetic variant is an essential but not sufficient condition for the occurrence of Gilbert syndrome. Patients with dehydration, starvation, fatigue, menstruating, or other diseases are at higher risk of unconjugated bilirubin elevation (Fretzayas et al., 2012; Memon et al., 2016).

Before the emergence of genetic testing, the diagnosis of Gilbert syndrome mainly depended on the exclusive diagnosis. For example, ruling out hepatobiliary diseases and hemolytic diseases, in combination with certain auxiliary examinations, such as the measurement of bilirubin after fasting test, administration of rifampin, and administration of phenobarbital, as well as measuring UGT enzyme activity by thin-layer chromatography, could facilitate the diagnosis. The global prevalence of Gilbert syndrome is about 3%–8.6%, and the common pathogenic mutations include A(TA)<sub>7</sub>TAA insertion (*UGT1A1*\*28) and p.Gly71Arg missense variant (*UGT1A1*\*6). The common mutation spectrum and mutation rate vary in different ethnicities. Previous studies have reported that p.Gly71Arg variant (about 15.7%) and A(TA)<sub>7</sub>TAA variant (9.7%) are the most common mutations in Japanese patients with Gilbert syndrome (Maruo et al., 2016) while in the Caucasian population, A(TA)<sub>7</sub>TAA variant (about 38.8%) is the most common mutation; however, p.Gly71Arg variant is the least occurring variant. A(TA)<sub>7</sub>TAA variant (about 44.6%) is also the only type reported in the African-American population, while p.Gly71Arg variant

is very rare (Kaniwa et al., 2005). Only very few studies reported the spectrum of *UGT1A1* variant in Chinese patients with Gilbert syndrome.

In the present study, we performed genetic testing of the *UGT1A1* gene in patients suspected of Gilbert syndrome and with the major manifestation of unconjugated bilirubin elevation. We furthermore investigated the features of the variant spectrum in Chinese patients confirmed with Gilbert syndrome. Several novel variants were also identified.

## 2 | METHODS

### 2.1 | Ethical compliance

Ethics approval was obtained from the ethics committee at Ruijin Hospital, Shanghai Jiao Tong University School of Medicine.

Clinical data and the whole blood samples of patients with hyperbilirubinemia who were treated in our hospital for unconjugated bilirubin elevation between 2015 and 2020 were collected, following the provision of informed consent by the patients. All the patients were Chinese Han people, and all received genetic testing of *UGT1A1*. The testing results showed that 117 patients met the diagnostic criteria for Gilbert syndrome, including 91 males (77.78%) and 26 females (22.22%), with the mean age of  $35.97 \pm 13.73$  years (7–68 years). The mean level of unconjugated bilirubin in patients was  $37.5 \pm 15.46$   $\mu\text{mol/L}$  (12.0–80.7  $\mu\text{mol/L}$ ).

A blood genomic DNA extraction kit (QIAamp<sup>®</sup> DNA Blood Mini Kit, Qiagen, Germany) was used to extract DNA from peripheral blood leucocytes, according to the previously reported methods (Gu et al., 2014). Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>) was used to design the polymerase chain reaction (PCR) primers. The promoter, 5 exons, and splicing sites of the *UGT1A1* gene were amplified. After terminal labeling, the PCR products were sequenced by ABI377 automatic DNA sequencer (Applied Biosystems, USA). The sequencing results were aligned to the reference DNA sequences of NG\_002601.2 in GenBank. The gene variants were named according to the nomenclature recommended by the Human Genome Variation Society (HGVS).

Serum levels of total bilirubin, conjugated bilirubin, and liver enzymes were also measured for all the patients who received genetic testing of *UGT1A1*. For the diagnosis of Gilbert syndrome, the patients needed to be with simple unconjugated hyperbilirubinemia, and serum level of unconjugated bilirubin  $\leq 85$   $\mu\text{mol/L}$ , while hepatocellular diseases (all patients received viral hepatitis serology screen, autoimmune antibodies, ceruloplasmin, and

ferritin measurement) and hemolytic diseases (patients received blood routine examination, reticulocytes, and haptoglobin measurement) were ruled out. All cases had no history of phenobarbital use and liver transplantation.

The known pathogenic variants were recorded and confirmed according to Human Gene Mutation Database (HGMD; <http://www.hgmd.cf.ac.uk/>) and the SNP database (dbSNP; <https://www.ncbi.nlm.nih.gov/snp/>). Novel variant refers to the variant not recorded in the relevant gene mutation databases or reported by the latest literature. The novel variants were input to online gene mutation prediction tools, including MutationTaster (<http://www.mutationtaster.org/>), and PROVEAN (<http://provean.jcvi.org/>) for pathogenicity prediction and potential splice impact. If tools predicted the pathogenicity, the novel variants were considered pathogenic, the classification of variants is then determined in combination with the ACMG (The American College of Medical Genetics and Genomics) classification rules (Richards et al., 2015). A pedigree chart was plotted for patients when possible.

### 3 | RESULTS

A total of 117 patients were confirmed with Gilbert syndrome by genetic testing of *UGT1A1*, most being young and middle-aged patients. All the patients were with slight elevation of unconjugated bilirubin (<85 μmol/L) on the diagnosis. These patients were with compound heterozygous mutation (64.1%) or homozygous mutation (35.9%), and the most common pathogenic variant was promoter A(TA)<sub>7</sub>TAA insertion (*UGT1A1*\*28), while another relatively common variant was the p.Gly71Arg missense variant (*UGT1A1*\*6) in the exon 1. The other less common pathogenic variants included p.Pro229Glu missense variant (*UGT1A1*\*27) in the exon 1, p.Pro364Leu and p.Arg403Cys missense variants in the exon 4, and p.Tyr486Asp missense variant in the exon 5 (Table 1). In our study, eight patients had both heterozygous p.Pro229Gln

and homozygous A(TA)<sub>7</sub>TAA variants, suggesting linkage disequilibrium between these two variants. These variants have been reported in previous studies on hereditary unconjugated hyperbilirubinemia, whose pathogenicity has also been demonstrated and recorded in HGMD.

Seven novel variants were identified by the genetic testing of *UGT1A1* in Gilbert syndrome patients (Table 2). None of these variants were reported or recorded before, and the pathogenicity was unclear. The pathogenicity was predicted by online genetic mutation prediction tools and classified according to ACMG classification rules (Richards et al., 2015) which showed that four out of the seven novel variants were pathogenic or likely pathogenic, and the other three were uncertain significance variants (Table 3). The four novel pathogenic variants included p.Ala61Gly missense variant and p.Leu166Alafs\*16 frameshift variant in the exon 1, p.Ser306Phe missense variant in the exon 2, and p.Glu424\* nonsense variant in the exon 4.

A 15-year-old male patient was found to have a novel p.Leu166Alafs\*16 frameshift variant. The patient and his parents all received genetic testing of the *UGT1A1* gene, and the pedigree chart was plotted (Figure 1). The findings showed that the patient was carrying compound heterozygous variants of promoter A(TA)<sub>7</sub>TAA and p.Leu166Alafs\*16, the mother was carrying a heterozygous variant of p.Leu166Alafs\*16, and the father was carrying a heterozygous variant of A(TA)<sub>7</sub>TAA. However, the serum bilirubin levels of the parents were normal. The pathogenicity of this novel variant was further clarified according to the online mutation prediction tool analysis results, as well as the clinical characteristics of the patient and the pedigree chart.

### 4 | DISCUSSION

Genetic testing of the *UGT1A1* gene is currently considered an important method for confirming Gilbert

TABLE 1 Sequence variants of the *UGT1A1* gene

Variants	Gene position	Base/repeat times		Het. no.	Hom. no.	Incidence (%)
		wt	Variants			
A(TA) <sub>n</sub> TAA	Promoter	6	7	51	36	52.56
p.G71R	211	G	A	53	17	37.18
p.P229Q	686	C	A	17	1	8.12
p.P364L	1091	C	T	13	1	6.41
p.R403C	1207	C	T	1	0	0.43
p.Y486D	1456	T	G	4	3	4.27

Note: Reference sequence: NG\_002601.2.

Abbreviations: Het. no., number of heterozygotes; Hom. no., number of homozygotes.

TABLE 2 Novel variants of the *UGT1A1* gene

Novel variants	Gene position	Base		Compound variants		Exon	Age (year)	Sex	UCB ( $\mu\text{mol/L}$ )
		wt	Variants	Allele 1	Allele 2				
p.A61G	182	C	G	p.G71R	N	1	28	F	26.9
p.Y67F	200	A	T	A(TA) <sub>7</sub> TAA	A(TA) <sub>7</sub> TAA	1	27	F	52.2
p.L166Afs*16	492_493	GT	del GT	A(TA) <sub>7</sub> TAA	N	1	15	M	51.3
p.R240K	719	G	A	p.G71R	N	1	29	M	23.7
p.S306F	917	C	T	A(TA) <sub>7</sub> TAA	N	2	35	F	27.5
p.R341Q	1022	G	A	p.G71R	N	3	41	M	28.6
p.E424*	1270	G	T	p.G71R	p.G71R	4	7	F	23.2

Note: Reference sequence: NG\_002601.2.

Abbreviations: F, female; M, male; N, normal; UCB, unconjugated bilirubin.

TABLE 3 Pathogenicity classification of novel variants

Novel variants	PROVEAN <sup>a</sup>		MutationTaster			ACMG Classification <sup>b</sup>
	SCORE	Prediction	Prob.	Prediction	Splice site	
p.A61G	-2.81	Deleterious	0.9996	Polymorphism	No abrogation	Likely pathogenic
p.Y67F	-0.77	Neutral	0.9999	Polymorphism	Changes	Uncertain significance
p.L166Afs*16	-3.49	Deleterious	1	Disease causing	Changes	Pathogenic
p.R240K	-0.53	Neutral	0.9999	Polymorphism	Changes	Uncertain significance
p.S306F	-4.59	Deleterious	0.9998	Disease causing	Changes	Likely pathogenic
p.R341Q	-0.17	Neutral	0.9999	Polymorphism	Changes	Uncertain significance
p.E424*	-8.44	Deleterious	0.9999	Disease causing	Changes	Pathogenic

Note: Reference sequence: NM\_000463.3 and NP\_000454.1.

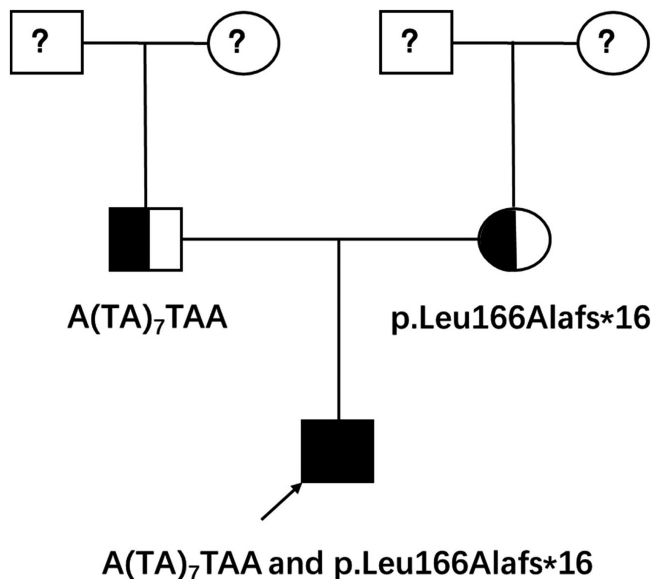
Abbreviation: Prob., probability.

<sup>a</sup>PROVEAN score cutoff = -2.5.

<sup>b</sup>Classification was based on available information that meets the ACMG criteria.

syndrome and Crigler–Najjar syndrome. As the prognosis is generally good, Gilbert syndrome does not require diet therapy or specific treatments. Genetic testing is mostly used in clinical practice to confirm the diagnosis, as it can alleviate patients' concerns and further provide genetic counseling, thus preventing the repeated hospitalization of patients. Undoubtedly, genetic testing of *UGT1A1* could also be used in the individualized treatment by some chemotherapeutic drugs (such as irinotecan; Sanchez-Dominguez et al., 2018). To date, more than 150 pathogenic variants of the *UGT1A1* gene have been identified, (Stevenson, 2020) where the most common ones include A(TA)<sub>7</sub>TAA and p.Gly71Arg variants. Our findings showed that A(TA)<sub>7</sub>TAA and p.Gly71Arg variants were also the most common variants in Chinese patients, which is consistent with the findings in other Asian populations (Maruo et al., 2016;

Sun et al., 2017; Wang et al., 2020). A(TA)<sub>7</sub>TAA variant is the insertion of an additional TA sequence in the TA repetitive sequence of the original A(TA)<sub>6</sub>TAA sequence in the promoter. Homozygous A(TA)<sub>7</sub>TAA variant could reduce *UGT1A1* gene expression by 70%, thus affecting the conjugation of bilirubin and leading to the elevation of unconjugated bilirubin in the peripheral circulation. While p.Gly71Arg variant is a common variant in the Asian population, it is very rare or even undetectable in the Caucasian and African populations. p.Gly71Arg variant is the transition of nucleotide 211 in exon 1 of the *UGT1A1* gene from G to A, resulting in the missense variant of the amino acid 71 from glycine to arginine. The heterozygous p.Gly71Arg variant could reduce the *UGT1A1* enzyme activity to 30%–60% (Long et al., 2011). p.Pro229Gln variant often co-occurred with the A(TA)<sub>7</sub>TAA variant, which is consistent with



**FIGURE 1** Pedigree chart of a Gilbert syndrome patient with novel p.Leu166Alafs\*16 mutation

the findings in some previous studies (Abuduxikuer et al., 2018; Canu et al., 2013; Sun et al., 2017). p.Pro364Leu variant was first discovered in a Taiwanese Gilbert syndrome patient (Huang et al., 2000). When compared with the wild-type, p.Pro364Leu variant reduced UGT1A1 enzyme activity to 64.4% (Takeuchi et al., 2004). p.Pro364Leu variant was found in previous studies involving Chinese, Japanese, and Indian patients with unconjugated hyperbilirubinemia, (Abuduxikuer et al., 2018; Farheen et al., 2006; Takeuchi et al., 2004) but not present in European patients. p.Tyr486Asp variant was first discovered in a Japanese patient with unconjugated hyperbilirubinemia (Aono et al., 1993). p.Tyr486Asp variant is in the exon 5, one of the shared exons, which may affect the enzyme activity of other members of the UGT1A subfamily (UGT1A6, UGT1A9; Court et al., 2001; Nakagawa et al., 2011).

In the present study, we identified seven novel variants by the genetic analysis of *UGT1A1*, where four variants, including p.Ala61Gly, p.Leu166Alafs\*16, p.Ser306Phe, and p.Glu424\*, might be pathogenic or likely pathogenic. A patient carrying the heterozygous p.Leu166Alafs\*16 variant also carried the heterozygous A(TA)<sub>7</sub>TAA variant. The patient's parents also underwent the testing of the *UGT1A1* gene, which showed that the parents also carried these two variants (Figure 1). Therefore, the pathogenicity of this novel variant was further confirmed by the pedigree analysis. Pathogenicity could not be completely ruled out for the other three uncertain significance novel variants. Detecting the structural and functional changes of proteins and enzyme activity after variant are reliable methods for confirming the pathogenicity of novel variants.

Nonetheless, further in vitro experiments are required to verify the pathogenicity of novel variants.

In summary, 117 patients were confirmed with Gilbert syndrome by genetic testing of the *UGT1A1* gene, revealing that A(TA)<sub>7</sub>TAA and p.Gly71Arg variants were common pathogenic variants in the Chinese population. In addition, we also identified seven novel variants, which expanded the spectrum of *UGT1A1* variants, and provided new references for relevant genetic diagnosis. However, future studies are needed further to investigate the pathogenic mechanisms of these novel variants.

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#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

#### AUTHOR CONTRIBUTIONS

Leilei Gu and Yue Han performed the genetic testing and contributed equally to this work. All the authors evaluated for clinical data and follow-up of the patients.

#### ETHICS APPROVAL

Ethics approval was obtained from the ethics committee at Ruijin Hospital, Shanghai Jiao Tong University School of Medicine.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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