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Prevalence of 2 *UGT1A1* Gene Variations Related to Gilbert's Syndrome in South of Iran: An Epidemiological, Clinical, and Genetic Study

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Abstract

Background: Gilbert's syndrome can present as a chronic or benign asymptomatic condition, characterized by a slight increase in the serum bilirubin level without any hemolysis. In 1995, a genetic variation, located in the TATA box of *UGT1A1* gene promoter, was identified in patients with Gilbert's syndrome. Also, further analysis identified a new missense variation, Gly71Arg, within the codon region of *UGT1A1* gene. Coincidence of TATA box and Gly71Arg variations and their relationship with clinical findings are mostly variable.

Objectives: The aim of this study was to determine TATA box and Gly71Arg variations of *UGT1A1* gene and assess their effects on clinical findings in patients with Gilbert's syndrome in southern provinces of Iran.

Methods: In this cross sectional study, 213 unrelated infants and children, below 12 years, who were admitted to the pediatric ward of Namazi hospital, Shiraz, Iran, were enrolled from June 2015 to May 2016. Blood-extracted DNA was used for genotyping TATA box and Gly71Arg variations by sequencing. Further biochemical analyses were performed for each patient.

Results: About 78.9% of the studied subjects had normal homozygous genotypes, and 21.1% were heterozygous for the Gly71Arg variation. In total, 34% of the cases were normal in the promoter region (TA6/6), and 55% were heterozygous with genotypes TA6/7, TA6/5, and TA 6/8. Three combinations of genotypes, ie, TA6/7-Gly/Gly, TA7/7-Gly/Gly, and TA7/7-Gly/Arg, showed significant differences in the serum total bilirubin level. Also, creatinine phosphokinase in TA6/7-Gly/Arg, TA7/7-Gly/Gly, and TA7/7-Gly/Arg had a significant increase.

Conclusions: The present findings showed that the TA7/7 promoter of *UGT1A1* gene accounted for a considerable number of Gilbert's syndrome cases (11.3%). The studied variations had a significant effect on creatine phosphokinase and serum total bilirubin levels.

Keywords: Bilirubin, Gilbert's Disease, TATA Box, Genetic Variation, Glucuronosyltransferase

1. Background

Gilbert's syndrome (GS), first described by Gilbert and Lereboullet, is an autosomal recessive disorder, caused by changes in uridine diphosphate (UDP) glucuronosyltransferase 1A1 (*UGT1A1*) gene (1). GS is characterized as a mild, unconjugated hyperbilirubinemia in the absence of hepatic disease and hemolysis (2). The main cause of increased serum bilirubin level in GS patients is the reduction of glucuronosyltransferase enzyme activity (3). In these patients, based on the genetic population makeup, UDP glucuronosyltransferase activity can be reduced by up to 30% of the normal level (2).

So far, researchers have performed comprehensive studies on different aspects of GS, thus leading to the better management and recognition of this condition (4). The main common genetic cause of GS is mutation in the TATA box of *UGT1A1* gene promoter, which plays a regulatory role in gene transcription. It is well known that 6 TA repeats (*UGT1A1**1) in this region are normal, while presence of 7 TA repeats (*UGT1A1**2) reduces glucuronosyltransferase activity; other variations are not well specified (5, 6).

It is speculated that the enlarged TATA box sequence is less effective in binding regulatory agents, which control the transcription of *UGT1A1* gene (7). Further studies revealed another cause of GS, ie, G> A variation at nucleotide 211 in exon 1, which results in the substitution of glycine

to arginine at codon 71 (Gly71Arg) of *UGT1A1* protein (8). In fact, the incidence of hyperbilirubinemia in neonates with Gly71Arg missense variation has been reported to be nearly 30% higher than the normal population in different ethnicities (9, 10). So far, 113 variants of *UGT1A* gene have been identified, which can lead to a wide spectrum of conditions ranging from jaundice and very mild conditions to severe, lethal diseases (11).

GS may not require a special medical treatment as it has benign consequences (12, 13). Although the main feature of GS is an elevated unconjugated bilirubin level, metabolism of some drugs and xenobiotics may be also affected (14). Acetaminophen, ethinylestradiol, indinavir, and irinotecan clearance from the serum is reduced in GS due to deficiency in drug glucuronidation. Accordingly, considering drug accumulation in the serum, some researchers have suggested a GS diagnostic test before irinotecan prescription to prevent severe side-effects (15-17).

Although all conducted studies have emphasized on the relationship between GS and increased bilirubin level, in the present study, in addition to confirming previous findings in this area, the relationship between GS and elevated creatine phosphokinase (CPK) level was analyzed. The present study aimed to determine 2 variations of *UGT1A1* gene and assess their effects on clinical findings in patients with GS.

2. Methods

2.1. Sample Collection

The sample size for this cross sectional study was determined to be 197, based on the following formula and previous studies (18, 19) (P = 0.09 and d = 0.04):

$$N = (196)^2 \frac{P(1-P)}{d^2} = 197 \quad (1)$$

To achieve this sample size, the process of subject recruitment started from June 2015. Any unrelated infant or child (below 12 years), who was referred to the emergency ward of Namazi hospital, Shiraz, Iran and was transferred to the pediatric ward, was included in this study. In May 2016, a total of 215 subjects were recruited, although 2 cases were eliminated, based on the exclusion criteria. Residence in Fars province or southern provinces of Iran and willingness to participate in the study were among the inclusion criteria.

Whole blood samples (1.5 mL) were collected from each patient in Greiner Bio-One Hematology K3-EDTA Lavender Evacuated Tubes and kept at -20°C until DNA extraction.

2.2. Ethical Considerations

Sample collection and subsequent analysis were performed after obtaining a written informed consent from each patient according to the Declaration of Helsinki. The study was approved by the Medical Ethics Committee on Human Research of Shiraz University of Medical Sciences (code number, IR.SUMS.rec.1394.S95).

2.3. Inclusion and Exclusion Criteria

The study population included children below 12 years, who were living in Fars province or southern provinces of Iran. On the other hand, all cases who used liver enzyme inducers, such as barbiturates, aminopyrine, phenylbutazone, orphenadrine, or 3, 4-benzpyrene, were excluded from the study (20). According to these criteria, among 215 included cases, 2 cases were eliminated.

2.4. Demographic and Biochemical Information

A demographic questionnaire (including name, gender, phone number, address, age, history of neonatal jaundice, parent's history of jaundice, and history of specific diseases) was completed for each subject. Liver function test (LFT), as well as CPK, lactate dehydrogenase (LDH), blood urea nitrogen (BUN), and creatinine (Cr) levels, was measured by MAN Co. kits with a CS-400 auto-chemistry analyzer (DIRUI Industrial Co., Ltd, Eastern China).

2.5. Preparation of Genomic DNA

Genomic DNA was extracted from the peripheral blood lymphocytes (100 - 200 μL) by the RIBO-prep nucleic acid extraction kit variant 100 (K2-9-Et-50-CE, Slovak Republic), according to the manufacturer's instructions. The concentration of the extracted genomic DNA was determined by measuring the ultraviolet absorbance at 260 nm with the NanoDrop Lite Spectrophotometer (Thermo Scientific, USA). About 70 - 110 ng of the genomic DNA was used for polymerase chain reaction (PCR).

2.5.1. PCR and DNA Sequencing

The promoter and exon 1 regions of UGT1A1 gene were amplified, using in-house designed primers. Forward and reverse primers to amplify the TATA box promoter and exon 1 included 5'-TGAAATTCCAGCCAGTTCAA-3' and 5'-TTGAAGACGTACCCTGTGC-3', respectively. The reaction mixture (25 μL) contained 1 μL of genomic DNA, 1 μL of each forward and reverse primer (10 pmol/reaction), 13 μL of AMPLIQON TEMPase Hot start 2x Master Mix (CinnaGen Co., Iran), and 9 μL of ddH_2O.

Briefly, PCR was performed by denaturation at 95°C for 15 minutes, followed by 35 cycles at 94°C for 40 seconds, 55°C for 40 seconds, and 72°C for 40 seconds, with a final

synthesis at 72°C for 5 minutes. The amplified PCR products were sequenced by forward and reverse primers, using the Sanger method (bi-directional sequencing). Finally, the nucleotide sequences were analyzed by BioEdit software.

2.6. Statistical Analysis

For data management and further analysis, SPSS version 21 was used. Descriptive analysis was performed for determining the frequency. Also, t test, Chi square, Fisher's exact test, and one-way analysis of variance (ANOVA), followed by Duncan's and LSD multiple-range tests, were used for pairwise comparisons. P value ≤ 0.05 was considered statistically significant. For data which were not normally distributed, we used nonparametric Kruskal-Wallis test, as well as Bonferroni correction test. In these tests, P value ≤ 0.003 was considered statistically significant.

3. Results

3.1. Demographic Characteristics

A total of 213 unrelated infants and children, below 12 years, were included in this study. The mean age of the subjects, including 123 males (57.75%) and 90 females (42.25%), was 5.69 $\pm$ 3.4 months. Among all cases, 25.3 % (n = 54) had a history of diseases. Overall, 61% (n = 130) of the patients' parents were consanguineously married. The mean body mass index (BMI) of the subjects was 16.36 $\pm$ 0.31 kg/m^2. Based on the findings, no relationship was found between these characteristics and the laboratory data. It should be also noted that all cases were from southern provinces of Iran.

3.2. Analysis of UGT1A1 Variations

In the evaluated population, 5 possible genotypes were identified, based on the amplification and sequencing of UGT1A1 gene promoter. These genotypes included common homozygous allele TA6/6, common heterozygous allele TA6/7, rare heterozygous alleles TA6/5 and TA6/8, and rare homozygous allele TA7/7. The minimum and maximum frequencies of alleles carrying TA variations were 0.5% and 53.5%, respectively (Table 1).

The genotype distribution of Gly71Arg variation among 213 Iranian children and neonates was determined as follows: Gly/Gly (n = 168, 78.9%) and Gly/Arg (n = 45, 21.1%) (Table 1 and Figure 1). With respect to the coincidence of promoter TATA box and Gly71Arg variations, 8 genotypes were detected. TA6/7-Gly/Gly was recognized as the most frequent genotype (46.1%), while TA6/8-Gly/Gly and TA6/5-Gly/Gly were rare genotypes (0.5%) (Table 2).

3.3. Laboratory Parameters

All the participants underwent LFT, CPK, LDH, BUN, and Cr tests. The results related to the association between laboratory tests and combined genotypes are presented in Tables 3 and 4. Among promoter and UGT1A1*6 variation groups, there was a statistically significant difference in the serum total bilirubin (STB) level between TA7/7-Gly/Gly (P = 0.017), TA7/7-Gly/Arg (P < 0.001), and TA6/7-Gly/Gly (P = 0.009) genotypes. Also, a statistically significant difference was found regarding CPK level between TA7/7-Gly/Gly (P = 0.034), TA7/7-Gly/Arg (P = 0.022), and TA6/7-Gly/Arg (P = 0.012) genotypes, compared to other groups. However, other liver laboratory tests showed no significant difference between the genotype groups.

Other than LDH test, gender had no effects on the results of laboratory tests in different genotype groups. Statistical analysis of UGT1A1*6 variations showed that homozygosity of Gly71Arg alone had no impact on the increase in STB or CPK level. The variation in LDH was significant in heterozygous Gly71Arg, while it was insignificant in combination with other variations in the promoter region. Also, we found no significant difference in STB level between children with and without a history of neonatal jaundice (0.53 $\pm$ 0.4; P = 0.9). Also, age and other demographic factors had no effects on liver laboratory test results.

4. Discussion

Although GS is known as a benign condition, it is of clinical importance due to its effects on the patient's liver enzyme profile. Clinical and epidemiological findings about GS in different ethnical groups can help researchers, pharmacists, and physicians introduce more effective medications. The prevalence of UGT1A1 gene polymorphisms in the promoter and exon 1 (Gly71Arg) regions has been separately reported in only a limited number of cases (21, 22). The present study revealed the coincidence of these 2 polymorphisms and the clinical manifestations in the Iranian population for the first time.

In this regard, Hemmati et al. (23) determined the prevalence of GS, using rifampin test in Fars province, south of Iran. They reported an incidence rate of 19.1% in both genders for GS in the general population. The results of their study showed that the prevalence of GS in males (25.6%) was higher than females (12.8%), which is inconsistent with the present findings (14.3% in males and 9% in females); also, the overall prevalence was much lower in the present study (11.3%). This discrepancy could be due to differences in the methods applied in these studies.

Table 1. Distribution of Promoter TATA Box and Gly71Arg Variants Based on Age and Gender

	Genotype Frequency (%)						
	Promoter TATA box					Gly/Arg	
Gender	TA6/6	TA6/7	TA6/8	TA6/5	TA7/7	Gly/Gly	Gly/Arg
Male	40 (32.8)	70 (56.6)	1 (0.8)	1 (0.8)	11 (9)	96 (78.7)	26 (21.3)
Female	33 (36.3)	45 (49.4)	0.0 (0.0)	0.0 (0.0)	13 (14.3)	72 (79.1)	19 (20.9)
Total (%)	73 (34.2)	114 (53.5)	1 (0.5)	1 (0.5)	24 (11.3)	168 (78.9)	45 (21.1)
Age[a]	37.7 ± 42.9	51.3 ± 47.0	12.0 ± 0.0	144.0 ± 0.0	49.9 ± 44.88	47.5 ± 46.38	42.0 ± 44.6

[a] Age in months (mean ± SD).

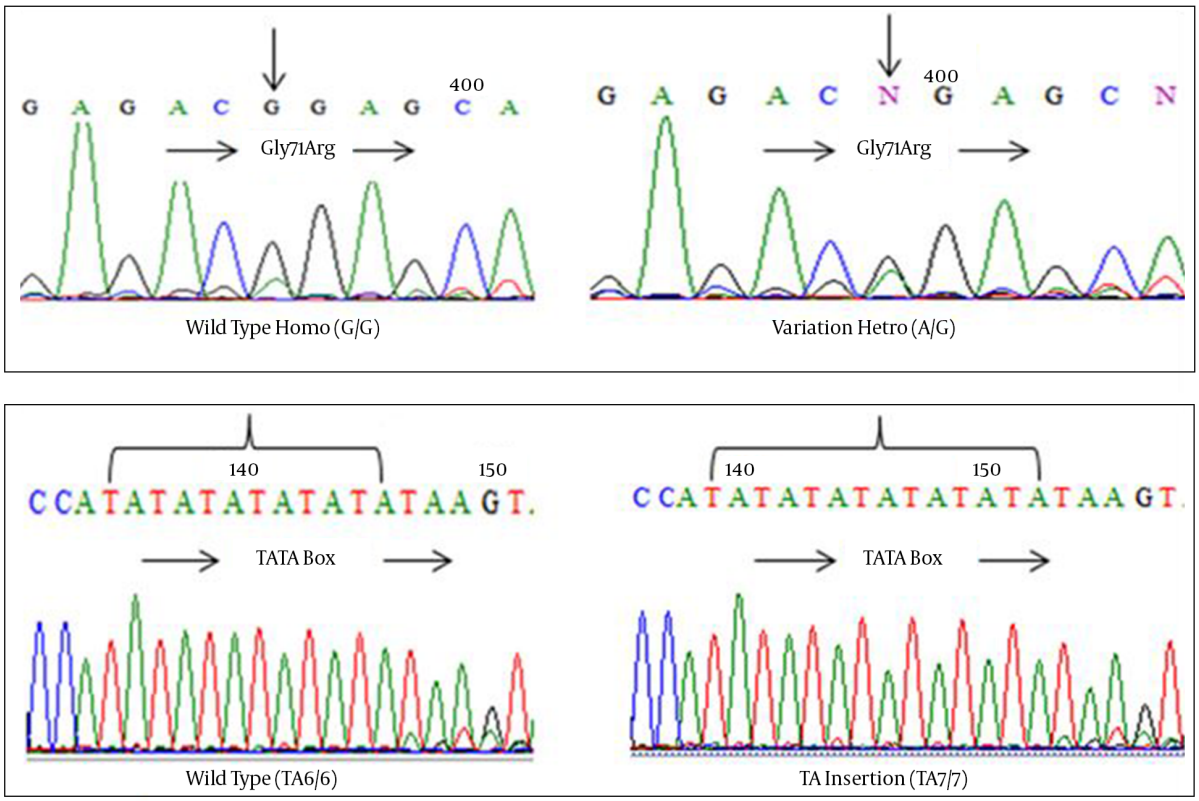

Figure 1. Sequencing chromatogram of *UGT1A1* gene in the c.211G > A site. Arrows indicate the nucleotide substitution position.

By using the restriction fragment length polymorphism (RFLP) method, Dastgerdy et al. found that the frequency of Gly71Arg variation (homozygous and heterozygous) was 30.4%, which was higher than the rate reported in the present study (21.1%). The high frequency might be attributed to the low specificity and sensitivity of RLFP method (24). Also, similar to studies by Ergin and Kaveh, we found that homozygous TA7/7 genotype is associated with increased STB level in case of additional TA insertion in the promoter (21, 25).

In the Malay population, Yossef et al. observed that the prevalence of homozygous TA7/7, heterozygous TA6/7, and heterozygous Gly71Arg (Gly/Arg) mutations was 7%, 18%, and 5.5%, respectively. In the present study, similar to the mentioned research, homozygous Gly71Arg (Arg/Arg) was not detected. However, we observed a higher frequency of heterozygous TA7/6 (55.4%) than homozygous TA7/7 (11.3%), compared to the study by Yusoff et al. (43.5% and 9.9%, re-

Table 2. Prevalence of Promoter TATA Box and Gly71Arg Variants Based on Gender in Southern Provinces of Iran

Genotype Ggroups TATA	Genotype Groups Gly71Arg	Gender				Total	
		Female		Male			
		Frequency	%	Frequency	%	Frequency	%
TA6/6	Gly/Gly	29	13.62	30	14.08	59	27.70
TA6/6	Gly/Arg	4	1.88	10	4.69	14	6.57
TA6/7	Gly/Gly	38	17.84	60	28.17	98	46.01
TA6/7	Gly/Arg	6	2.82	10	4.69	16	7.51
TA7/7	Gly/Gly	4	1.88	5	2.35	9	4.23
TA7/7	Gly/Arg	9	4.23	6	2.82	15	7.04
TA6/5	Gly/Gly	0	0	1	0.47	1	0.47
TA6/8	Gly/Gly	0	0	1	0.47	1	0.47
Total		90	42.25	123	57.75	213	100

Table 3. The Results of Laboratory Tests (Mean & IQR) in the Genotype Groups[a]

Laboratory test (unit)	Genotype Groups															
	TA6/6 Gly/Gly		TA6/6 Gly/Arg		TA6/7 Gly/Gly		TA6/7 Gly/Arg		TA7/7 Gly/Gly		TA7/7 Gly/Arg		TA6/5 Gly/Gly		TA6/8 Gly/Gly	
	Median	IQR[b]	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
ALP (U/L)	300.0	171.8	275.5	213.0	300.0	116.5	318.0	232.0	369.0	51.9	314.0	60.5	756.0	0.0	422.0	0.0
AST (U/L)	41.0	31.5	36.0	48.0	38.0	38.5	47.0	49.5	69.0	108.5	53.0	94.3	47.0	0.0	19.0	0.0
ALT (U/L)	24.0	30.0	21.5	24.3	19.5	47.1	24.0	55.0	20.0	114	19.0	50.0	23.0	0.0	8.0	0.0
LDH (U/L)	490.0	897.0	580.0	665.5	522.0	366.5	895.5	1571.3	1586	4049	478.0	238.4	48.0	0.0	427.0	0.0
BUN (mg/dL)	9	9.5	8.0	6.8	12.0	8.0	11.0	6.3	8.0	9.5	11.5	6.8	7.0	0.0	6.0	0.0
Cr (mg/dL)	0.5	0.30	0.5	0.35	0.5	0.2	0.6	.35	0.5	0.3	0.5	0.2	4.1	0.0	0.2	0.0

Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BUN, blood urea nitrogen; Cr, creatinine; LDH, lactate dehydrogenase.
[a] The difference between the wild type and other genotypes is significant ($P \leq 0.05$).
[b] Interquartile range (IQR) = Q3 - Q1.

Table 4. The Results of Laboratory Tests (mean ± SD) in the Genotype Groups

Laboratory test (unit)	Genotype Groups							
	TA6/6 Gly/Gly	TA6/6 Gly/Arg	TA6/7 Gly/Gly	TA6/7 Gly/Arg	TA7/7 Gly/Gly	TA7/7 Gly/Arg	TA6/5 Gly/Gly	TA6/8 Gly/Gly
STB (mg/dL)	0.40 ± 0.20	0.41 ± 0.18	0.54± 0.21[a]	0.46 ± 0.32	0.85 ± 0.12[a]	0.87 ± 0.21[a]	0.40 ± 0.00	0.20 ± 0.00
CPK (U/L)	358.20 ± 219.2	132.00 ± 80.0	204.58 ± 194.30	444.40 ± 134.14[a]	469.00 ± 83.20[a]	452.50 ± 9.7[a]	127.00 ± 0.00	114.00 ± 0.00

Abbreviations: BUN, blood urea nitrogen; CPK, creatine phosphokinase; Cr, creatinine; STB, serum total bilirubin.
[a] The difference between wild type and other genotypes is significant ($P \leq 0.05$).

spectively) (26, 27). Also, Nettles et al. showed that 11% of the Swiss population had GS, and the polymorphism had a significant correlation with the total bilirubin level. The present findings were in line with the results reported by Nettles et al.; nevertheless, they did not study other liver enzymes (28).

The results of the present study showed that Gly71Arg variation had no independent effects on STB level; this indicates that this variation only affects neonatal jaundice, not bilirubin level in the postneonatal period (9, 10). We found that none of the 2 variations (TATA and Gly71Arg) alone had a significant effect on CPK level, while in combi-

nation with some other genotypes, an increase in CPK level was revealed.

In some studies, the prevalence of TA7/7 and Gly71Arg variations has been reported to be 3 - 19% and 20 - 60%, respectively (29-33). One of the important findings of the present study was identifying 2 rare TA6/8 and TA6/5 genotypes with no significant increase in STB level. These genotypes have been previously reported in African populations (34). In the current study, we also showed a significant increase in the CPK level in GS cases, which has not been previously reported; nevertheless, for further clarification, more studies are required.

The strengths of the present study include the use of sequencing method, detection of 2 rare mutations (TA6/8 and TA6/5), and the relationship between GS and CPK level. On the other hand, the shortcoming of this study was the limited sample size due to restricted sources. In conclusion, the present study showed that the prevalence of GS in south of Iran was nearly similar to other populations.

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Footnote

Conflicts of Interest: The authors declare no conflicts of interest.

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