IJN
Iranian Journal of Neonatology
http://ijn.mums.ac.ir

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Original Article

Genetic Association of *UGT1A1* Promoter Variants (c.-3279T>G and c.-3156G>A) with Neonatal Hyperbilirubinemia in an Iranian Population

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ABSTRACT

Background: Several studies have reported that two promoter variants (c.-3279T>G and c.-3156G>A) in UDP-glucuronosyltransferase (*UGT1A1)* gene may contribute to neonatal hyperbilirubinemia. However, these variants have not been investigated in Iranian neonates. This cross-sectional study aimed to determine if the *UGT1A1* promoter variants are significant risk factors associated with neonatal hyperbilirubinemia.

Methods: A total of 178 unrelated neonates, including newborns with neonatal jaundice (n=95) and healthy controls (n=83), were included in this study. Each individual was genotyped by the PCR-RFLP and COP-PCR at nucleotides -3279 and -3156, respectively, using fresh blood DNA. Logistic regression analyses were performed to assess the association of *UGT1A1* promoter variants with the presence of significant hyperbilirubinemia. Anthropometric indices and clinical variables were also compared between the different genotype groups.

Results: Allele and genotype analysis of the c.-3279T>G and c.-3156G>A variants showed no significant association with the risk of neonatal hyperbilirubinemia neither in the crude nor after adjustment for gestational age, gender, and birth weight in different genetic models (P>0.05). However, in haplotype-association analysis, only one haplotype (A-T) was found to be associated with the risk of neonatal hyperbilirubinemia (OR=0.19, 95% CI; [0.18–0.20], P=0.001).

Conclusion: This study failed to demonstrate that c.-3279T>G and c.-3156G>A variants alone might contribute to the risk of neonatal hyperbilirubinemia in Iranian neonates. However, the A-T haplotype may play a significant role in increasing the risk of hyperbilirubinemia.

Keywords: Hyperbilirubinemia, Kernicterus, Polymorphism, UGT1A1

Introduction

Neonatal hyperbilirubinemia is one of the most common causes of hospitalization in newborns. Approximately, 60% of term and 80% of preterm infants develop neonatal jaundice in the first week after birth (1). A growing body of evidence has shown that different ethnic populations represented various prevalence and severity of neonatal jaundice. East Asian population is more susceptible and presents a greater tendency for severe jaundice than Caucasians (2-4). Serum total bilirubin concentration at any time point represents a regulated balance between bilirubin production and elimination from the body. Any positive imbalance leads to the accumulation of bilirubin that generates a pathogenic condition called hyperbilirubinemia.

In healthy newborns, it is known that nonpolar, water-insoluble, unconjugated bilirubin is converted into a more polar and water-soluble substance using uridine diphospho (UDP)-glucuronate-glucuronosyltransferase1 A1 (*UGT1A1*) (5). This is an enzyme responsible for bilirubin glucuronidation, which is encoded by the *UGT1A1* gene located on chromosome 2q37 consisting of

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Please cite this paper as:
Pour Alizadeh N, Mamouri G, Boskabadi A, Boskabadi H, Rafatpanah H, Moradi A, Mehrad-Majd H. Genetic Association of *UGT1A1* Promoter Variants (c.-3279T>G and c.-3156G>A) with Neonatal Hyperbilirubinemia in an Iranian Population. Iranian Journal of Neonatology. 2021 Apr: 12(2). DOI: 10.22038/ijn.2021.50368.1884

four common exons (2, 3, 4, and 5) and 13 variable exons (6, 7). Any reduction in the enzymatic activity of *UGT1A1* results in the accumulation of unconjugated hyperbilirubinemia.

The role of genetic factors has been recognized as significant in maintaining the levels of serum TB within physiological or abnormal ranges (8, 9). Mutations in the *UGT1A1* gene, especially in variable exon A1, promoter, or common exons 2 to 5, have been considered a possible genetic risk factor for neonatal hyperbilirubinemia (10). These mutations may affect the enzyme structure and function; moreover, it causes failure in bilirubin conjugation with subsequent jaundice. In addition, mutations in the coding region of the *UGT1A1* gene have shown associations with different disorders in bilirubin metabolism, hemolysis, and bilirubin transport (11).

Various variants located in promoters, such as 211G>A and A $(TA)_7$TAA, have also been reported to be frequent in newborns with neonatal hyperbilirubinemia in East Asian populations (2-4, 12-21). A large body of evidence has demonstrated a possible association between two common *UGT1A1* promoter polymorphisms (A $(TA)_7$TAA and c.211G>A) in various populations (13, 14). However, few studies have examined the potential role of the other two c.-3279T>G and c.-3156G>A variants during the development of neonatal hyperbilirubinemia (14, 20, 22). Recently, two studies have been conducted on two different Iranian infant populations with or without neonatal jaundice to evaluate the genotype association of A $(TA)_7$TAA and c.211G>A variants with neonatal hyperbilirubinemia (23, 24). To the best of our knowledge, the 3279T>G and c.-3156G>A polymorphisms have not yet been examined in the Iranian population, and there is little information about the genetic susceptibility to neonatal hyperbilirubinemia in Iranian neonates. Therefore, it is hypothesized that these two polymorphisms of c.-3279T>G and c.-3156G>A might act as risk factors for neonatal hyperbilirubinemia in the Iranian population. Accordingly, the current cross-sectional study was conducted to elucidate their possible genotype association with neonatal hyperbilirubinemia in an Iranian neonate population.

Methods

Patients and controls

A total of 178 unrelated Iranian infants with gestational age>38 weeks and birth weight >2500 gr were enrolled in this study from December 2016 to February 2018 in Ghaem Hospital, Mashhad, Iran. Written informed consent was obtained from all parents, and the study protocol was approved by the Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran. The subjects were divided into two groups of healthy neonates without jaundice as the control group (n=83) and icterus neonates as the case group (n=95). All these neonates were free of risk factors, such as blood group and Rh incompatibility, G6PD deficiency, maternal diabetes, polycythemia, infection, asphyxia, hypothermia, hypoglycemia, cephalohematoma, or liver dysfunction. Neonatal hyperbilirubinemia was defined based on the American Academy of Pediatrics guidelines and as an hour-specific total serum bilirubin risk nomogram (25). All affected neonates were treated for neonatal hyperbilirubinemia with phototherapy or exchange transfusion. The control group consisted of neonates with serum TB levels below the 40th percentile according to Bhutani nomogram (6). Serum TB levels and G6PD enzyme activity were measured by the spectrophotometric method as described previously (26).

Determination of UTG1A1 gene variants

Genomic DNA was extracted from the whole blood of the neonates using a blood DNA isolation Kit (Genet-bio) according to the manufacturer's protocol. The quality of the DNA samples was assessed by agarose gel electrophoresis, and the concentration was quantitated by NanoDrop1000. To genotype the c.-3279T>G variant, polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) was carried out using the forward (5-CACCAGAACAAACTTCTGAG-3) and reverse (5-GTTCTCAAATTGCTTTGTTTA-3) primers covering the specific site for restriction enzyme *DraI.* The PCR-amplification was carried out with an Astec Gradient thermocycler (Tokyo, Japan) in a final volume of 25 µl containing 20 ng of genomic DNA under the following conditions: an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 52°C for 45 sec, extension at 72°C for 45 sec, and a final extension at 72°C for 7 min. The resulting PCR products were checked on 2% agarose gel and then digested with *DraI* at 37°C for 18 h. The mutant G allele lacks the restriction site (141 bp); however, the PCR product containing the wild T allele was cleaved to a 121-bp and a 20-bp fragment.

To screen for the c.-3156G>A mutation, a combination method of semi-nested PCR and competitive oligonucleotide priming (COP)-PCR was applied according to a study conducted by

Yusoff et al. (20). Briefly, two rounds of PCR were performed. In the first round, the target region of the *UGT1A1* promoter was amplified using a set of two common forward and reverse primers. In the second round, COP-PCR was conducted with the common forward primer used in the first round PCR and a reverse primer, which specifically binds to one of the variant nucleotides (Inner for -3156A (5'-CTGTCAAAGCTC-3') or inner for -3159G (5'-CTGTCCAAGCTC-3'). For genotyping quality control, 5% of the samples were measured in duplicates and nuclease-free water was used as a negative control.

Statistical analysis

All statistical tests were performed using SPSS software (version 22.0) (SPSS Inc., Chicago, IL). Genotypic and allelic frequencies as well as the association between the genetic polymorphisms and the risk of neonatal hyperbilirubinemia were assessed using the Chi-square test. Moreover, the differences among the means in demographic, anthropometric, and biochemical parameters were evaluated by the Student's t-test, ANOVA, and posthoc multiple comparisons. Odds ratios with 95% confidence interval (CI) adjusted for gestational age, birth weight, and gender were determined by comparing each genotype (heterozygous, homozygous) to the wild type through binomial logistic regression analysis. The pairwise D and r^2 values for linkage disequilibrium and haplotype frequencies for two genetic variants were estimated using online cube X software (27). A p-value less than 0.05 was considered statistically significant.

Results

Clinical and demographic characteristics

Table 1 summarizes the demographic and baseline clinical characteristics of cases and controls. No significant differences were found between the cases and controls regarding the male: female ratio, as well as mean birth weight and gestational age. Regarding feeding, 77.9% of neonates in the hyperbilirubinemia group were exclusively breastfed, and 3.2% of cases were exclusively formula-fed. Moreover. 18.9% of newborns were fed on a mixture of breast milk and formula. On the other hand, 72.3% of neonates in the control group were exclusively breastfed, and the remaining (27.7%) were fed on a mixture (P=0.223). Furthermore, there was a significant difference between the cases and controls in terms of delivery method. About 79% and 52% of neonates in the hyperbilirubinemia and control groups were born via vaginal delivery, respectively (P<0.001).

UGT1A1c.-3279T>G and c.-3156G>A genotypes and allele distribution

The genotype distributions, allele frequencies, and association between the two *UGT1A1* promoter variants and the risk of hyperbilirubinemia are listed in Table 2. No deviation from Hardy-Weinberg equilibrium was observed in genotype distributions for c.-3279T>G variant just in the control group (P=0.303). Regarding the c.-3279T>G polymorphism, the frequencies of TT, TG, and GG genotypes were 25.3%, 60%, and 14.7% in the hyperbilirubinemia group, as well as 39.8%, 50.6%, and 9.6% in the control group, respectively. The genotype distributions of the c.-3279T>G variant were not significantly different between the two groups (P=0.103).

No significant differences were also found between the cases (65%T and 35% G) and controls (55%T and 45% G) (P=0.06) in terms of allelic frequencies of this variant. There was also no significant difference in the genotype distribution of the c.-3156G>A variant between the cases and controls (P=0.612). Briefly, the GG, GA, and AA frequencies were 38.9%, 58.9%, and

Table 1. Characteristics of the neonates with and without hyperbilirubinemia.

Characteristics	Case (n=95)	Control (n = 83)	P-value
M/F	55/40	51/32	0.951
Gestational Age (weeks)	38.41 ± 4.03	38.99 ± 1.09	0.311
Birth Weight (gr)	2964.2 ± 441.3	3099.7 ± 469.1	0.572
STB (mg/dl)	18.31 ± 6.17	9.04 ± 3.66	0.001
Delivery Type	-	-	-
Vaginal	75 (79.0)	43 (52.0)	< 0.001
Cesarean	20 (21.0)	40 (48.0)	
Types of feeding	-	-	-
Exclusive breastfeeding	74 (77.9)	60 (72.3)	
Exclusiveformulafeedig	3 (3.2)	0 (0.0)	0.223
Mixed feeding	18 (18.9)	23 (27.7)	

M/F, male/female; STB, Serum total bilirubin; Values were expressed as mean ±SD, P-value Cases vs. Controls

Table 2. Genotype distribution and allele frequencies of the *UGT1A1* polymorphisms in infants with and without hyperbilirubinemia.

	Cases (n=95)	Controls (n=83)	Adjusted OR[a] (95% CI)	[a]*P*
c.-3279T>G Genotype, n (%)				
TT	24 (25.3)	33 (39.8)	1.00	-
GT	57 (60.0)	42 (50.6)	0.61 (0.29-1.29)	0.544
GG	14 (14.7)	8 (9.60)	0.44 (0.15-1.35)	0.690
[b]*P* = 0.103				
T allele	105 (55.0)	108 (65.0)	1.00	
G allele	85 (45.0)	58 (35.0)	1.55 (0.93-3.33)	0.060
[b]*P*= 0.610				
c.-3156G>A Genotype, n (%)				
GG	37 (38.9)	38 (45.8)	1.00	
GA	56 (58.9)	44 (53.0)	0.82 (0.41-1.60)	0.554
AA	2 (2.2)	1 (1.2)	0.67 (0.06-8.02)	0.750
GA + AA	58 (61.0)	45 (54.2)	0.81 (0.41- 1.60)	0.542
[b]*P*= 612				
G allele	130 (68.4)	120 (72.0)	1.00	-
T allele	60 (31.6)	46 (28.0)	1.20 (0.76-1.90)	0.426
[b]*P* = 0.426				

OR, odds ratio; CI, confidence interval. P> 0.05: Not significant
[a]*P* of Logistic regression models adjusted for gestational age, gender, and birth weight.
[b]*P* of X^2test related to allelic and genotypic differences between cases and controls.

2.2% in cases, as well as 45.8%, 53.0%, and 1.2% in controls, respectively. There was also no significant difference between the two groups regarding rare allele c.-3156A frequency (P=0.426).

The crude odds ratios were also assessed in this study for the risk of hyperbilirubinemia in subjects carrying the rare alleles or different genotypes of these two variants by logistic regression in different genetic models. The results failed to reveal any evidence of a significant increase or decrease in the risk for hyperbilirubinemia (Table 3). Moreover, no significant changes were observed in previous results after adjusting for gender, birth weight, and gestational age (Table 3).

Diplotype and haplotype analysis in the UGT1A1 promoter region

To evaluate the combined effect of the two

Table 3. Logistic regression analysis for genotype distribution and allele frequencies of the *UGT1A1* polymorphisms in different genetic models

Variant	Model	Genotype	Case	Control	Crude OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
c.-3279T>G	Codominant	TT	24 (25.3)	33 (75)	1.00	0.10	1.00	0.27
		TG	57 (60)	42 (24)	0.54 (0.28-1.04)		0.61 (0.29-1.29)	
		GG	14 (14.7)	8 (1)	0.42 (0.15-1.15)		0.44 (0.15-1.35)	
	Dominant	TT	24 (25.3)	33 (39.8)	1.00	0.04	1.00	0.13
		TG-GG	71 (74.7)	50 (60.2)	0.51 (0.27-0.97)		0.58 (0.28-1.19)	
	Recessive	TT-TG	81 (85.3)	75 (90.4)	1.00	0.30	1.00	0.33
		GG	14 (14.7)	8 (9.6)	0.62 (0.25-1.55)		0.62 (0.23-1.66)	
	Overdominant	TT-GG	38 (40)	41 (49.4)	1.00	0.16	1.00	0.47
		TG	57 (60)	42 (50.6)	0.68 (0.38-1.24)		0.79 (0.41-1.52)	
	Log-additive	---	---	---	1.09 (0.62-1.92)	0.77	0.65 (0.0.38-1.11)	0.11
Variant	Model	Genotype	Case	Control	Crude OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value
c.-3156G>A	Codominant	GG	37 (39.0)	38 (45.0)	1.00		1.00	
		GA	56 (59.0)	44 (53.0)	0.76 (0.42-1.9)	0.61	0.82 (0.41-1.60)	0.82
		AA	2 (2.1)	1 (1.2)	0.49 (0.04-5.60)		0.67 (0.06-8.02)	
	Dominant	GG	37 (37.0)	38 (32.0)	1.00	0.36	1.00	0.54
		GA-AA	58 (63.0)	45 (68.0)	0.76 (0.42-1.3724)		0.81 (0.41-1.59)	
	Recessive	GG-GA	93 (97.9)	82 (98.8)	1.00	0.64	1.00	0.43
		AA	2 (2.1)	1 (1.2)	0.57 (0.05-6.37)		0.77 (0.07-8.83)	
	Overdominant	GG-AA	39 (41.0)	47 (44.0)	1.00	0.43	1.00	0.59
		GA	56 (59.0)	53 (56.0)	0.79 (0.43-1.42)		0.83 (0.43-1.62)	
	Log-additive	---	---	---	0.75 (0.43-1.33)	0.33	0.82 (0.43-1.54)	0.53

Table 4. Diplotype and haplotype frequencies for the *UGT1A1* gene in the neonatal hyperbilirubinemia group and control subjects

Haplotype	Frequency among cases	Frequency among controls	OR (95% CI)	P	Diplotype	N
G-T	0.51	0.64	-	-	TT – GG	53 (29.9)
A-G	0.28	0.27	0.67 (0.33-1.36)	0.27	TT – X/A	3 (0.017)
G-G	0.17	0.08	0.57 (0.32-1.03)	0.07	X/T – GG	22 (0.12)
A-T	0.03	0.01	0.19 (0.18-0.20)	0.001	X/T – X/A	99 (0.6)
		Disequilibrium				
D'	0.780	0.960				
r^2	0.35	0.66				

The degree of linkage disequilibrium between the two variants is shown as D' for each group.

variants on the risk of hyperbilirubinemia, cases and controls were divided into four groups according to the combination of the two genotypes from each SNP (TT-GG, TT-XA, XT-GG, and XT-XA) (Table 4). There was a significant difference between the infants with hyperbilirubinemia and those in the control group in terms of diplotype distribution (P=0.033). In fact, the frequency of T and G carriers was significantly higher in controls, compared to the cases, supporting a protective role for the co-expression of T and G alleles against the risk of hyperbilirubinemia in neonates (Table 4).

The haplotype frequencies of these two polymorphisms upstream in the *UGT1A1* promoter and the degree of linkage disequilibrium by D' and r^2 were also computed in both case and control groups (Table 4). The obtained results showed that the two polymorphisms were in strong linkage disequilibrium (D'=0.780, r^2=0.35 in cases, and D'=0.960, r^2=0.66 in controls). Furthermore, haplotype analysis for the two *UGT1A1* variants revealed four possible haplotypes, including G-T, A-G, G-G, and A-T, with an estimated frequency of more than 1% in both groups. It is worth noting that there was a significant difference between the two study groups in terms of haplotype distribution. Compared to the most common haplotype (G-T), only the A-T haplotype was associated with a significantly increased risk of hyperbilirubinemia (OR=0.19, 95% CI= 0.18-0.20, P=0.001).

Discussion

This cross-sectional study investigated the possible association between c.-3279T>G and c.-3156G>A polymorphisms in the *UGT1A1* gene and the risk of hyperbilirubinemia in Iranian neonates. Previous studies (n=2) have considered the association of two common A $(TA)_7TAA$ and c.211G>A polymorphisms in *UGT1A1* with hyperbilirubinemia in two different Iranian infant populations with or without neonatal jaundice; however, no previous study has investigated this association regarding the c.-3279T>G and c.-3156G>A polymorphisms (23, 24).

Therefore, the present study is the first to simultaneously consider the association and any possibility of c.-3279T>G and c.-3156G>A interaction regarding the hyperbilirubinemia in Iranian neonates. The obtained results revealed no statistically significant differences between the neonates with and/or without hyperbilirubinemia both in allelic frequency and genotype distribution of two c.-3279T>G and c.-3156G>A polymorphisms. The existing data indicated that the frequency of occurrence of the c.-3279T>G allele variant is highly variable among different ethnicities and showed a different range in Indians (87%), Caucasians (47%), and Japanese (25%) (28-30). In contrast to previous findings, 3279T>G mutation in our study occurred at a relatively lower rate at 14.7% in the hyperbilirubinemia group which was similar to controls. This finding is in line with the finding of an earlier Taiwanese report (31); however, it was inconsistent with the results of previous studies on Indian (14) and Egyptian neonate populations (22).

This discrepancy can be attributed to the racial differences and variable sample sizes in different studies. An almost equal distribution of the mutant allele and genotype was also found for the c.-3156G>A polymorphism which was in line with the results of the study performed by Silva et al. (14).

However, given that these two polymorphisms are in close linkage disequilibrium in the *UGT1A1* gene, a significant difference in diplotype and haplotype distribution was observed in infants with hyperbilirubinemia, compared to the control subjects. The neonates who carried A-T haplotype were more prone to develop hyperbilirubinemia. This is in line with the findings of the previous reports, which suggested the mutant haplotype as a risk factor for the increased prevalence of neonatal hyperbilirubinemia (14, 32).

The findings of the studies that examined the possible association of c.-3279T>G and c.-3156G>A variants with the risk of neonatal

hyperbilirubinemia in different populations are contradictory. At least one reason for this discrepancy can be attributed to the race and ethnicity depended on the varied extent of linkage disequilibrium (D') between the two variants in different populations (33). In our study, the observed D' amount was in its strongest value (D'=0.8 and D'=0.96 in case and controls, respectively), indicating a synergistic effect for the combination of these two polymorphisms. Accordingly, studies that have examined each of these two variants separately have led to different outcomes.

Conclusion

The findings showed no possible association between the *UGT1A1* gene c.-3279T>G and c.-3156G>A variants and the risk of neonatal hyperbilirubinemia in their solitary form. However, when considered together, there was a significant potential role for the increased risk of hyperbilirubinemia, indicating the possibility of the interaction between these two variants regarding the increased risk of jaundice. Further studies are suggested to be conducted with larger sample sizes on diverse ethnic populations to support evidence for these results.

Acknowledgments

This study was supported by the research project No. 951554 as a neonatology residency thesis at Mashhad University of Medical Sciences, Mashhad, Iran. The authors would like to thank the Clinical Research Development Unit and Department of Pediatrics at Ghaem Hospital, Mashhad, Iran, for their assistance in this research.

Conflicts of interest

The authors declare no conflicts of interest regarding the publication of this study.

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