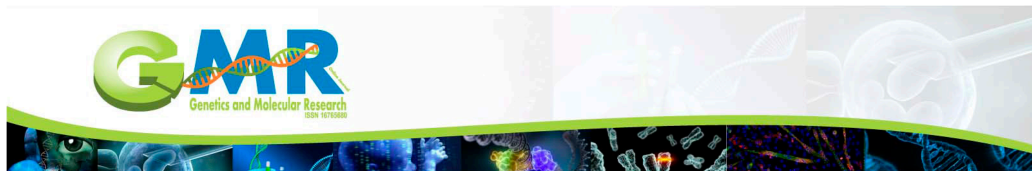

Analysis of uridine diphosphate glucuronosyl transferase 1A1 gene mutations in neonates with unconjugated hyperbilirubinemia

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ABSTRACT. This study was carried out to analyze uridine diphosphate (UDP)-glucuronosyltransferase 1A1 (*UGT1A1*) gene mutations in neonates with unconjugated hyperbilirubinemia, from two different ethnic groups. Polymerase chain reaction and gene sequencing were used to analyze the differences in genotypes and allele frequencies of different gene mutations among the ethnic groups; this was followed by checking their correlation with the serum bilirubin level and the occurrence of unconjugated hyperbilirubinemia in neonates. Our results reveal that the *UGT1A1* mutant genotype, 211G>A, is distributed differently in the case *vs* control groups, as well as in the Zhuang *vs* Han ethnic groups. Moreover, this difference is statistically significant ($P < 0.05$); the total serum bilirubin (TSB) and unconjugated bilirubin (UCB) levels in patients carrying the single homozygous mutation, 211G>A, were markedly higher than that in patients without the mutation ($P < 0.05$). Furthermore, the TSB and UCB levels were significantly different between patients carrying single or compound

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211G>A heterozygous mutation, (TA)6/7, and 1941C>G/2042C>G heterozygous mutation, and patients without mutation ($P > 0.05$). Our findings suggest that the 211G>A mutation in the first exon may be a risk factor for unconjugated hyperbilirubinemia in Zhuang and Han neonates. The serum bilirubin levels seem to be affected by the homozygosity or heterozygosity of the *UGT1A1* gene mutation; 211G>A homozygous mutation is an important factor that causes a rise in bilirubin in neonates with unconjugated hyperbilirubinemia.

Key words: Unconjugated hyperbilirubinemia; Neonate; UGT1A1; Gene mutation

INTRODUCTION

In neonates, bilirubin is derived from heme decomposition. Nonpolar and liposoluble unconjugated bilirubin (UCB), which cannot be discharged via urine, is transported to the liver through the blood brain barrier in combination with albumin in the blood plasma. By combining with the Y and Z proteins (Zhang et al., 2007; Kalkan et al., 2014), UCB forms water-soluble conjugated bilirubin (CB) in the smooth endoplasmic reticulum, under catalysis by uridine diphosphate (UDP)-glucuronosyltransferase 1A1 (UGT1A1). Hyperbilirubinemia can be further categorized as unconjugated hyperbilirubinemia, conjugated hyperbilirubinemia, and mixed hyperbilirubinemia, based on the increase in total serum bilirubin (TSB), UCB, and CB. Unconjugated hyperbilirubinemia in neonates, resulting from an increase in UCB, is a common health issue worldwide (Shenoi and Nandi, 1997; Ihara et al., 2003).

UGT1A1, a member of the UGT1 supergene family, is located on chromosome 2 (2q37). Its 5' end includes 13 replaceable UGT1A1 in the first exon and 3' end covers four common exons (2nd to 5th exon) of the UGT1 gene. The promoter region upstream of the transcription start site in the first exon contains a TATA box sequence, and the total length of the coding region is 1602 bp (Long et al., 2011; Canu et al., 2013). Allelic differences in UGT1A1 among the Chinese population are expected because China has many ethic groups nationalities. Our results show that not all UGT1A1 mutations are associated with the occurrence of unconjugated hyperbilirubinemia in China; hence, it will be worthwhile to systematically analyze the correlation between different types of mutations in different populations and unconjugated hyperbilirubinemia, in various parts of the world.

MATERIAL AND METHODS

Research subjects

A total of 320 neonates (weighing 2.5-4 kg) of the Zhuang and Han ethnicities aged 3-15 days were selected from the Department of Pediatrics, Binzhou People's Hospital (Shandong) from June 2012 to June 2014. Before case collection, family members were informed in detail about our research and the possible risks involved, and their signed consent was obtained and recorded. The medical ethics committee of Binzhou People's Hospital approved the performance of this clinical trial in the hospital.

The neonates were divided into unconjugated hyperbilirubinemia group and control

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group, with 160 neonates (Zhuang and Han) in each group. Total bilirubin value of neonates in the unconjugated hyperbilirubinemia group was high (95%) while the value in the control group was less than 40% according to the percentile-based hour specific bilirubin nomogram (Berk et al., 1969).

Neonates were excluded if they had congenital malformation, hemolytic disease resulting from ABO or Rh incompatibility, glucose-6-phosphate dehydrogenase (G6PD) deficiency, low birth weight (LBW), anoxia, asphyxia, macrosomia, septicemia, cephalhematoma, abnormal liver function, trisomy 21, or polycythemia vera.

Experimental materials

The following equipment was used: Table top High Speed Micro Centrifuge H1650-W (Yuqiang Technology Co., Ltd., Chengdu, China), DYY-2C bistable electrophoresis apparatus (Bajiu Industrial Co., Ltd., Shanghai, China), DYCP-31DN electrophoresis tank (Southeast Yicheng Laboratory Equipment Co., Ltd., Guangdong, China), ABI 9700 thermocycler (Aiyan Biological Technology Co., Ltd., Shanghai, China), VeritiTM 96-well gradient PCR amplifier (McGonagall Trade Co., Ltd., Beijing, China), SZ-93 automatic dual pure water distiller (Southeast Yicheng Laboratory Equipment Co., Ltd.), and Gel doc XR + imaging system (Bio-Rad, Shanghai, China).

The following reagents were used: blood genome small-quantity pillar extraction kit (Kanglang Biological Technology Co., Ltd., Shanghai, China), ethylenediaminetetraaceticacid (EDTA) (Jianglai Biological Technology Co., Ltd., Shanghai, China), Tris (hydroxymethyl) aminomethane (Tris) (Yuanmu Biological Technology Co., Ltd., Shanghai, China), boric acid (Huamaike Biotechnology LLC, Beijing, China), agarose (Ziqi Biological Technology Co., Ltd., Shanghai, China), absolute ethyl alcohol (Senbeijia Biological Technology Co., Ltd., Nanjing, China), 5X tetrabromoethane (TBE) buffer (Yaoyun Biological Technology Co., Ltd., Shanghai, China), primers (Yaoyun Biological Technology Co., Ltd.), GoldView nucleic acid stain (Haoran Biological Technology Co., Ltd., Shanghai, China), 2X Taq Mastermix (Kanglang Biological Technology Co., Ltd.), and DM2000 DNA marker (CWBIO Co., Ltd., Beijing, China).

Experimental methods

Peripheral venous blood (2 mL) was extracted and EDTA was added to it for anticoagulation. Then, the water bath was set at 37°C.

A blood genome minimum extraction kit, collection tube, and adsorption column were used for DNA processing. One milliliter of whole blood and 1 mL rat basophil leukemia cell (RBL) buffer were mixed in a 2-mL centrifuge tube and centrifuged at 137 *g* for 2 min; then, the supernatant was aspirated and the cell nuclear precipitate remained at the bottom of the tube. The above step was repeated once to completely decompose the cells. Glutathione reductase (GR) buffer (200 μL) and protease (20 μL) were successively added to the precipitate and mixed, followed by the addition of 200 μL GL. After that, the solution was reversed and mixed several times during incubation (10 min, 56°C). Absolute ethyl alcohol (200 μL) was added and mixed 5 times, and then pulse centrifuged. The obtained solution was transferred to the adsorption column and centrifuged for 1 min at 137 *g*. The supernatant was discarded and the adsorption column was put back and centrifuged again for 2 min at 137 *g*. Again, the supernatant was discarded and the adsorption column was placed at room temperature for a

few minutes until it dried completely. Then, the adsorption column was placed into another centrifuge tube (1.5 mL) along with 100 μL GE buffer, placed at room temperature for 5 min, and then centrifuged at 137 *g* for 1 min to collect the DNA solution. This step was repeated once to increase the DNA yield. The collected DNA was stored for further use only if the concentration was over 100 ng/μL and OD_{260}/OD_{280} was between 1.7 and 1.9, as measured by ultraviolet spectrophotometry. Finally, it was preserved at - 20°C.

The following primers were synthesized: Exon1N1 primer sequence F: AATGGATCC TGAGGTTCTGG; R: CTGGGTAGCCTCAAATTCCA, with the length of 834 bp; Exon1N2 primer sequence F: TTGTCTGGCTGTTCCCACTT; R: TGCCAAAGACAGACTCAAACC, with the length of 657 bp; Exon2 primer sequence F: AACACGCATGCCTTTAATCATA; R: TGACAACAACCACAACAACAAA, with the length of 416 bp; Exon3 primer sequence F: GAAGTTGCCAGTCCTCAGAAG; R: TGTTGGCCATAATATTTTCAAGC, with the length of 356 bp; Exon4 primer sequence F: AACACTGAGTCTTTGGAGTGTTTTC; R: TATTTGA AACAACGCTATTAAATGCT, with the length of 420 bp; Exon5N1 primer sequence F: CAG GTTTCCTTTCCCAAGTTT; R: GGGGGCACGATACATATTCA, with the length of 625 bp; Exon5N2 primer sequence F: AATTAATCAGCCCCAGAGTGC; R: GAAGGCGTGTGTGT GTGAAC, with the length of 750 bp.

The required reagents were thawed, mixed and pulse centrifuged, and put on ice for PCR amplification. The 50-μL reaction mixture included 25 μL 2X Taq Mastermix (CWBIO, Beijing, China), 1 μL upstream and downstream primers (Table 1), and 2 μL template DNA; deionized water was added to reach the final 50-μL volume, and the solution was mixed gently and pulse centrifuged. The PCR products were separated by gel electrophoresis and DM2000 DNA marker was used to verify their sizes. The DM2000 DNA marker comprises the following six DNA fragments: 2000, 1000, 750, 500, 250, and 100 bp; a bright band corresponding to the expected molecular weight was considered as the target band. Fuzzy target bands resulting from occasional factors, non-target bands, or bands mixed with additional PCR products were eliminated, and PCR was repeated until the target bands were observed. The PCR products in the target bands were sequenced to identify the gene mutation loci, based on comparisons with known sequences. The presence of the mutation was confirmed by reverse transcription, for accuracy.

Statistical methods

SPSS16.0 software was used for statistical analysis; data are reported as means ± standard deviation (SD). Data were analyzed using *t*-test (two independent samples), and numerical data, genotype distribution, and allele frequency were compared using the chi-square test or the Fisher exact test. Odds ratio (OR) of the influence of the mutation on neonatal unconjugated hyperbilirubinemia at 95% confidence interval (CI), were analyzed using binary variable logistic regression. Differences were considered to be statistically significant if $P < 0.05$.

RESULTS

Comparison of neonates from case and control groups

Table 1 shows the comparison between neonates from the case group and control group. The differences in the number of gestational weeks, birth weight, genders, and feeding patterns between the two groups were not statistically significant ($P > 0.05$).

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Table 1. Comparison of the general features of neonates from different groups.

General information	Zhuang		P	Han		P
	Case group	Control group		Case group	Control group	
Gestational weeks	39.1 ± 1.0	39.3 ± 1.0	0.053	39.1 ± 1.1	39.0 ± 1.2	0.647
Birth weights	3.1 ± 0.5	3.1 ± 0.3	0.013	3.1 ± 0.3	3.1 ± 0.4	0.972
Male/female	46/34	42/38	0.647	40/40	57/23	0.058
Breast feeding/non-breast feeding	68/12	69/11	0.689	58/22	71/9	0.813

Types and distribution of the UGT1A1 gene mutations

The following nine types of mutations were detected in the subjects: promoter TATA box (TA)7 insertion, 189C>T, 211G > A, and 686C>A in the first exon; and 1352C>T, 1459C>T, 1491C>T, 1941C>G, and 2042C>G in the fifth exon (Figure 1).

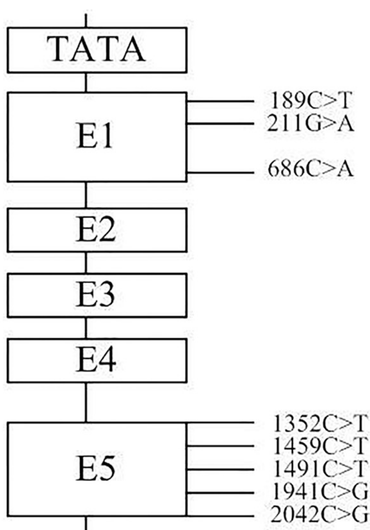

Figure 1. Location of the *UGT1A1* gene mutations in Zhuang and Han neonates.

The order of the nine mutation types (from high to low) in the case group, in terms of their frequencies, is as follows: 211G>A, (TA)7 insertion, 1941C>G, 2042C>G, 189C>T, 1491C>T, 686C>A, 1352C>T, and 1459C>T; among them, 1941C>G and 2042C>G always occur together, 1459C>T occurs only in Zhuang neonates, and 686C>A and 189C>T occur only in Han neonates (Table 2).

Table 2. Distribution of the different types of *UGT1A1* mutations.

Mutation types	Zhuang		Han	
	Case group (No. of cases)	Control group (No. of cases)	Case group (No. of cases)	Control group (No. of cases)
(TA)7 insertion mutation	18	20	14	23
211G>A	22	7	33	19
686C>A	-	-	1	-
189C>T	-	-	2	-
1352C>T	-	1	1	-
1459C>T	1	-	-	-
1491C>T	1	1	1	-
1941/2042C>G	8	9	10	10

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Genotype distribution and allele frequency of the UGT1A1 211G>A mutation

As shown in Table 3, comparison of the case *vs* control groups shows that the 211G>A mutation is distributed differently among the two groups and this difference is statistically significant (P < 0.01). Comparison of the case or control groups of the Zhuang *vs* Han ethnic groups, reveals that the 211G>A mutation is also distributed differently among the ethnic groups and this difference is statistically significant (P < 0.05). Although the case group shows higher frequency of the A allele than the control group in both ethnic groups, the difference is more pronounced in Han group than in Zhuang group (P < 0.01).

Table 3. Comparison of the genotype distribution of *UGT1A1* 211G>A mutation in each group.

Ethnicity	Groups	No. of cases	A/A (No. of cases)	G/A (No. of cases)	G/G (No. of cases)	P	A (%)	G (%)	P
Zhuang	Case group	80	3	20	57	<0.001	15.5	84.5	<0.001
	Control group	80	0	7	73	0.043	4.5	95.5	<0.001
Han	Case group	80	9	24	47	0.003	26	74	<0.001
	Control group	80	0	19	61	0.006	12	88	0.009

Genotype distribution and allele frequency of UGT1A1 1941C>G/2042C>G mutation

As shown in Table 4, the difference between genotype distribution and allele frequencies of 1941C>G/2042C>G mutation was not statistically significant (P > 0.05); similarly, there was no significant difference between the case groups or control groups of Han and Zhuang ethnic groups (P > 0.05).

Table 4. Comparison of the genotype distribution and allele frequency of *UGT1A1* 1941C>G/2042C>G mutation.

Ethnicity	Groups	Number of cases	C/G (No. of cases)	C/C (No. of cases)	P	G (%)	C (%)	P
Zhuang	Case group	80	8	72	0.804	5	95	0.816
	Control group	80	9	71	0.501	5.5	94.5	0.520
Han	Case group	80	10	70	0.917	6.5	93.5	0.941
	Control group	80	9	71	0.748	6.2	93.8	0.759

Influence of UGT1A1 mutation on neonatal unconjugated hyperbilirubinemia

By logistic regression analysis, we found that the UGT1A1 211G>A mutation affected Zhuang and Han neonates with hyperbilirubinemia, with OR (95%CI) of 3.946 (1.757, 8.841) and 2.219 (1.143, 4.255), respectively (P < 0.05). This finding suggests that 211G>A mutation is a risk factor for hyperbilirubinemia. Besides, Zhuang and Han neonates carrying 211G>A mutation (G/A or A/A) were 3.946 and 2.219 times more likely to develop hyperbilirubinemia, compared with their wild type (G/G) counterparts. (TA)7 insertion affected the development of hyperbilirubinemia in Zhuang and Han neonates, with OR (95%CI) of 0.854 (0.442, 1.618) and 0.057 (0.247, 1.030), respectively. 1941C>G/2042C>G mutation had an influence on hyperbilirubinemia in Zhuang and Han neonate, with OR (95%CI) of 0.916 (0.353, 2.215) and 1.052 (0.432, 2.519), respectively (P > 0.05). Details are displayed in Table 5.

Influence of UGT1A1 mutation on serum bilirubin levels

Eight three-day-old patients were divided into groups based on their genotypes; the average serum bilirubin concentration in each group is shown in Table 6. Only one patient

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carrying a single 211G>A homozygous mutation had markedly higher TSB and UCB levels than that in patients without the mutation (‘non mutation’ group). The average TSB and UCB levels in patients harboring single or compound 211G>A, (TA)7, and the 1941C>G/2042C>G heterozygous mutation were not statistically different from that of the ‘non mutation group’ ($P > 0.05$).

Table 5. Influence of different types of *UGT1A1* mutations on neonatal unconjugated hyperbilirubinemia.

Mutation sites	Ethnicity	Genotypes	No. of cases in case group	No. of cases in control group	OR (95%CI)	P
211G>A	Zhuang	G/G	58	73	3.946 (1.757-8.841)	0.001
		G/A+A/A	22	7		
	Han	G/G	47	61	2.219 (1.143-4.255)	0.015
		G/A+A/A	33	19		
TATA	Zhuang	(TA)6/6	62	60	0.854 (0.442-1.618)	0.620
		(TA)6/7+(TA)7/7	18	20		
	Han	(TA)6/6	66	57	0.057 (0.247-1.030)	0.061
		(TA)6/7+(TA)7/7	14	23		
1941C>G/ 2042C>G	Zhuang	C/C	72	71	0.916 (0.353-2.215)	0.823
		C/G	8	9		
	Han	C/C	69	70	1.052 (0.432-2.519)	0.946
		C/G	11	10		

Table 6. Comparison of serum bilirubin levels between different *UGT1A1* mutation groups and ‘non mutation’ group.

Group	Number of cases	TSB (μM)	P	UCB (μM)	P
Non mutation	32	255.7 ± 42.73		210.64 ± 46.18	
211G>A homozygous (A)	6	301.43 ± 52.74	0.036	275.86 ± 63.50	0.005
(TA)7/7	1	278.48	-	220.19	-
211G>A heterozygous (B)	16	262.06 ± 30.47	0.418	234.81 ± 45.83	0.121
(TA)6/7(C)	9	264.38 ± 12.87	0.527	227.76 ± 34.93	0.273
1352C>T heterozygous	1	237.26	-	228.25	-
1941/2042C>G heterozygous (D)	5	247.13 ± 22.61	0.689	224.38 ± 31.06	0.483
A+189C>T heterozygous	1	324.32	-	309.44	-
A+D	1	250.34	-	205.73	-
B+C	3	259.72 ± 16.83	0.872	201.47 ± 9.68	0.739
B+D	4	253.20 ± 23.15	0.846	216.05 ± 47.5	0.761
C+D	1	251.49	-	236.88	-

DISCUSSION

The 211G>A mutation, i.e., the G - A mutation in the 211th nucleotide in the first exon of *UGT1A1*, causes arginine to replace glycine at position 71 (G71R) of the corresponding protein; this is known as a missense mutation (Hagiwara et al., 1994; Tseng et al., 2010). The frequency of 211G>A mutation (A/A + G/A) in the case group was 34.38%, with the A/A homozygous genotype accounting for 7%. Among Zhuang patients, the 211G>A mutation was found in both case group (28%) and control group (9%), and the frequencies of the ‘A’ allele in the two groups were 0.155 and 0.045%, respectively. Among Han patients, the mutation was found in both case group (41%) and control group (23.6%), and the frequencies of the ‘A’ allele in the two groups were 0.26 and 0.119%, respectively. By comparing Zhuang and Han patients in the case group or control group, we found that Han patients have a significantly higher frequency of the mutation and A allele, than Zhuang patients do. The finding that A allele frequency in the case group is strikingly higher than that in the control group (regardless of ethnicity) indicates that the *UGT1A1* 211G>A mutation is associated with the occurrence

of unconjugated hyperbilirubinemia in Zhuang and Han neonates. Together with our results from the logistic regression analysis, *UGT1A1* 211G>A mutation seems to be a risk factor for unconjugated hyperbilirubinemia in Zhuang and Han neonates. Research in several parts of Asia, such as Korea, Japan, and Thailand, have led to the same conclusion as the one reported in China (Sappal et al., 2002; Kanai et al., 2005; Narter et al., 2011; Wanlapakorn et al., 2015), whereas studies in most parts of Europe and America showed no correlation of the 211G>A mutation with the occurrence of neonatal unconjugated hyperbilirubinemia in the region (Akaba et al., 1998; Ferraris et al., 2006).

(TA)7 insertion mutation refers to insert one more TA sequence based on (TA) 6 so as to transform normal A(TA)6/6TAA mutation into A(TA)6/7TAA hybrid subtype and A(TA)7/7TAA homozygotic type (Fernández Salazar et al., 2000). The present study showed that both TATA box genotype distribution and (TA) 7 allele frequency are not significantly different between either case *vs* control groups or between Zhuang *vs* Han population (either case or control group).

1941C>G and 2042C>G were located in 3'-untranslated region (UTR), with the minimum allele frequency (MAF) of 0.1768 and 0.2392 and allele frequency of 0.0578 and 0.0578 respectively. Hanchard et al. detected 1941C>G and 2042C>G in 178 cases of children with jaundice in Minnesota, and the G allele frequency was found to be 0.17 and 0.21 respectively, which is higher than what we have reported in this study (Hanchard et al., 2011). We found that 1941C>G and 2042C>G are unrelated to neonatal unconjugated hyperbilirubinemia in Zhuang and Han populations; moreover, there is no obvious influence on the serum TSB and UCB levels. This might be because they are not functionally mutated or have no linkage relationship with functional mutations; hence, they have no or little influence on the stability of mRNA.

Four other point mutations were detected, *viz.*, 189C>T, 1352 C>T, 1459C>T, and 1491C>T; their MAFs in the SNP database were 0.0009, 0.0009, countless value, and 0.0005, respectively, and the allele frequencies were 0.0026, 0.0026, 0.0013, and 0.0039, respectively. Only one 189C>T heterozygous mutation was detected in 181 cases of neonatal unconjugated hyperbilirubinemia (Skierka et al., 2013). 189C>T(Asp→Asp) and 1491C>T(Ala→Ala) are synonymous mutations that do not result in an amino acid change, while 1352C>T and 1459C>T are missense mutations that lead to an amino acid change (1352 C>T: Pro→Leu; 1459C>T: His→Tyr). They occur at very low frequencies, and their influence on enzyme expression and their pathological significance remains to be further explored (Tesapirat L et al., 2015; Chen et al., 2012).

To sum up, 211G>A mutation in the first exon is likely to be a hot spot of UGT1A1 gene mutation in Zhuang and Han neonates with unconjugated hyperbilirubinemia. The serum bilirubin level may vary depending on the homozygosity or heterozygosity of the UGT1A1 gene mutation; 211G>A homozygous mutation is an important factor resulting in bilirubin increase in unconjugated hyperbilirubinemia neonates. There are some caveats in this study, which could not be addressed due to certain limitations with respect to experimental conditions; currently, we are in the process of making the necessary improvements to achieve perfection in future studies.

Conflicts of interest

The authors declare no conflict of interest.

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