Relationship Between Bilirubin UDP-Glucuronosyl Transferase 1A1 Gene and Neonatal Hyperbilirubinemia

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ABSTRACT

The variation rate within the coding region of UDP-glucuronosyl transferase 1A1 (*UGT1A1*) gene in Taiwan Chinese was found to be 29.3%. This study sought to determine whether that high variation rate of *UGT1A1* gene is a risk factor for neonatal hyperbilirubinemia. The study subjects consisted of 123 newborn infants suffering from unconjugated hyperbilirubinemia who had no known risk factors for hyperbilirubinemia and 218 healthy control neonates. The promoter area, exons 1 to 4, coding region of exon 5, and the flanking intronic regions in *UGT1A1* gene were determined by the PCR in all subjects. Wild *UGT1A1* gene, variation in the promoter, variation at nucleotide 211, variation at nucleotide 1091, and compound heterozygous variation of *UGT1A1* gene were found. The percentage of neonates with wild *UGT1A1* gene and the percentage of neonates with variation at nucleotide 211 were significantly different between the study subjects and controls. The percentages with bilirubin $\geqq 342$ μM (20.0 mg/dL) and with persistent hyperbilirubinemia in the subjects carrying homozygous variation at nucleotide 211 (Gly71Arg) were significantly higher than the neonates carrying wild type or other genotypes. In conclusion, this study has demonstrated that variation at nucleotide 211 of the *UGT1A1* gene is a risk factor for the development of neonatal hyperbilirubinemia. Pediatricians should closely follow hyperbilirubinemic newborn infants who carry homozygous 211 G to A variation in *UGT1A1* gene. (*Pediatr Res* **52: 601–605, 2002**)

Abbreviations
UGT1A1, UDP-glucuronosyl transferase 1A1
CN, Crigler, Najjar
G6PD, glucose, 6-phosphate dehydrogenase
6/7, A(TA)$_6$TAA/A(TA)$_7$TAA

UDP-glucuronosyl transferase 1A1 (UGT1A1) is the key enzyme for bilirubin conjugation. Defects in this enzyme can cause a nonhemolytic unconjugated hyperbilirubinemia such as Crigler-Najjar syndrome type 1 (CN 1), type 2 (CN 2) and Gilbert's syndrome. Because UGT1A1 is too labile to be measured by classic biochemical methods, nonhemolytic unconjugated hyperbilirubinemia is ideally studied at the genetic level (1). The cDNA of human *UGT1A1* gene was found to be located at chromosome 2q37 and was cloned in 1991 (1). This led to the detection of genetic defects in patients with CN 1 (2), CN 2 (3), and Gilbert's syndrome (4). CN 1 and Gilbert's syndrome have been shown to be mainly associated with mutations in exons 2 to 5 and promoter area, respectively (5).

CN 2 and Gilbert's syndrome may be inherited as a recessive trait or as a dominant trait (4, 6, 7).

The peak serum levels of unconjugated bilirubin in full-term Asian (Japanese, Korean, or Chinese) and American Indian neonates are double those in Caucasian and black populations (8). The incidence of kernicterus is also higher among Asian newborn infants (9). These findings suggest that genetic factors are involved in the development of neonatal hyperbilirubinemia. Recently, homozygous A(TA)$_7$TAA variation in promoter of *UGT1A1* gene was found to be associated with neonatal hyperbilirubinemia in Sephardic Jews (10), Americans (11), Italians (12, 13), and British (14). However, in Japanese studies, the high allele-frequency of Gly71Arg in *UGT1A1* gene was found to be responsible for neonatal hyperbilirubinemia without obvious cause (15, 16). These reports reveal that the relationship between the site of variant *UGT1A1* gene and neonatal hyperbilirubinemia may differ among ethnic groups.

Very recently, we used the technique of PCR to determine the *UGT1A1* gene in Taiwan Chinese adults and found that the

occurrence of A(TA)$_7$TAA allele was relatively rare (14.3% *versus* 40%) and the variation rate within the coding region was much higher (29.3% *versus* 0.1%) in Taiwan Chinese compared with Caucasians (17). In addition, we found a novel compound heterozygous variation within the coding region of the *UGT1A1* gene that caused CN 2 in an adult patient (18). We hypothesized that the variations in the coding region of the *UGT1A1* gene may be related to the development of neonatal hyperbilirubinemia. We performed this study to determine whether this association existed. To the best of our knowledge, this study is the pioneering research of *UGT1A1* gene in Chinese neonates.

METHODS

Study subjects and healthy controls. Neonatal hyperbilirubinemia was diagnosed if a newborn infant needed phototherapy and had a bilirubin level $\geq$ 256.5 μM (15.0 mg/dL) in serum (8, 19) within 1 week after birth. From August 1999 through October 2000 at Cathay General Hospital, Taipei, Taiwan, 289 neonates (174 males and 115 females) suffered from neonatal hyperbilirubinemia. In addition, 25 neonates (14 males and 11 females) with hyperbilirubinemia were referred for investigation from National Taiwan University Hospital, Taipei, Taiwan. Totally 314 newborn infants were enrolled and the known risk factors for neonatal hyperbilirubinemia were investigated. Among the 314 newborn infants with neonatal hyperbilirubinemia, 191 had at least one known risk factor for the development of this disease (Table 1). The most frequent risk factor was breast milk feeding, followed by premature birth, ABO incompatibility, hypoxia/asphyxia, dehydration/vomiting, glucose-6-phosphate dehydrogenase (G6PD) deficiency, cephalohematoma, sepsis, and low birth weight. Some subjects had multiple risk factors (two or three factors) as shown in Table 1. The remaining 123 neonates (73 males and 50 females) had nonhemolytic unconjugated hyperbilirubinemia without obvious risk factors for hyperbilirubinemia. They were selected as the study group and were followed for 3 weeks. A bilirubin level $\geq$ 342 μM (20.0 mg/dL) was considered an indication for blood exchange (19). Persistent hyperbilirubinemia was defined as a bilirubin value over 150 μM (8.8 mg/dL) at day 14 of life (14). Control subjects (130 males and 88 females) were selected from full-term neonates born at Cathay General Hospital who had no known risk factors for neonatal hyperbilirubinemia, had peak bilirubin values <256.5 μM (15.0 mg/dL) and were apparent healthy during the 1st week of life. All the parents of study and control neonates gave consent to let their babies participating in this study that was approved by the research committee of Cathay General Hospital.

Analysis of the **UGT1A1** *gene.* The blood samples for the analysis of the *UGT1A1* gene were obtained from umbilical cord blood. Umbilical cord blood samples have been routinely collected for screening of G6PD deficiency in neonates at Cathay General Hospital since 1981 (19, 20). Total genomic DNA was isolated from whole blood cells using the blood DNA isolation kit (Maxim Biotech Inc, San Francisco, CA, U.S.A.). Exons 1 to 4 and coding region of exon 5 of the *UGT1A1* gene, their flanking intronic regions, and the promoter area were amplified by PCR as previously described (17). The primers used for PCR are shown in Table 2. The amplification reaction mixture (100 μL) contained 1 μg DNA in 10 mM Tris-HCl pH = 8.8, 1.5 mM MgCl$_2$, 50 mM KCl, 0.1% Triton X-100, 200 μM of each dNTP, 100 ng of each primer, and 2U of Dynazyme DNA polymerase (Finnzymes OY, Espoo, Finland). The reaction was performed with a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, U.S.A.) as follows: three cycles of denaturation at 94°C for 80 s, annealing at 55°C for 60 s and primer extension at 72°C for 110 s; seven cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 110 s; 30 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 50 s, and extension at 72°C for 90 s; and a final extension step at 72°C for 10 min. These PCR products were sequenced with an automated fluorescence sequencer (ABI Prism377, PE Applied Biosystems, Foster City, CA, U.S.A.), according to the manufacturer's instructions.

Statistical analysis. Student's *t* test and χ^2 test (with or without Yates' correction) were used to analyze the data as appropriate. A *p* value less than 0.05 was defined as statistically significant.

RESULTS

Male/female ratio, gestation age and birth weight. Table 3 shows the comparisons of male/female ratio, gestation age, and birth weight between study and control groups. There were no significant differences observed.

UGT1A1 *gene in newborn infants.* As shown in Table 4, wild *UGT1A1* gene, variation in promoter (A(TA)$_6$TAA/A(TA)$_7$AA (6/7) and A(TA)$_7$TAA/A(TA)$_7$TAA), variation at nucleotide 211 (G to A, heterozygous and homozygous), variation at nucleotide 1091, and compound heterozygous variation of *UGT1A1* gene were all identified among the study and control subjects. The percentage of the neonates carrying wild *UGT1A1* gene and the percentage of subjects having the variant nucleotide 211 were significantly different between the study and control neonates. The various allele frequencies of *UGT1A1* gene are listed in Table 5. Significant difference in the frequency of 211 G to A was found between the study subjects and the controls, while the frequencies of A(TA)$_7$TAA, 686 C

Table 1. *Risk factors for neonatal hyperbilirubinemia in 191 newborn infants*

Risk factor	n
Breast milk feeding	38
Premature birth	36
ABO incompatibility	31
Hypoxia/asphyxia	25
Dehydration/vomiting	9
G6PD deficiency	8
Cephalohematoma	6
Sepsis	3
Low birth weight	1
Two risk factors	30*
Three risk factors	4†

Four subjects * and three subjects † were G6PD deficient.

Table 2. *Primers used for PCR and sequencing of UGT1A1 gene*

Region	Primer			Target size (bp)
		Name	Sequence	
Promoter	PCR:	UPF1	5′GAGTATGAAATTCCAGCCAG3′	495
		UPR1	5′ACTCTTTCACATCCTCCCTT3′	
	Sequencing:	UPR2	5′ATGGCACAGGGTACGTCTTC3′	
Exon 1	PCR:	U1F1	5′AGATACTGTTGATCCCAGTG3′	495
		U1R1	5′GCACGTAGGAGAATGGGTTG3′	
	Sequencing:	U1F2	5′GAGGGGACATGAAATAGTTG3′	
	PCR:	U1F3	5′CAGCTTTGATGTCATGCTGA3′	497
		U1R2	5′CGATCCAAAGTAATACATCTG3′	
	Sequencing:	U1F4	5′CACTGTATTCTTCTTGCATG3′	
Exon 2	PCR:	U2F1	5′ATTCTGTAAGCAGGAACCCT	475
		U2R1	5′TAATAGTTGGGAAGTGGCAG3′	
	Sequencing:	U2F2	5′CTCTATCTCAAACACGCATG3′	
Exon 3	PCR:	U3F1	5′ATAGTTCTGCATCCACTTGTT3′	585
		U3R1	5′TGTTACTCACATGCCCTTGC3′	
	Sequencing:	U3F2	5′CCTTCACAGTTACTGATCCT3′	
Exon 4	PCR:	U4F1	5′GGCTTAAGCACAGCTATTCT3′	495
		U4R1	5′CATGAATGCCATGACCAAAGT3′	
	Sequencing:	U4F2	5′GCCAACATATCCTACATTGC3′	
Exon 5	PCR	U5F1	5′TGCAGTTAGCCATGCTTGTG3′	560
		U5R1	5′GGAAATGACTAGGGAATGGT3′	
	Sequencing:	U5F2	5′GTAGTCTTCTTAAGCAGCCA3′	

Table 3. *Basic data of newborn infants with hyperbilirubinemia and healthy controls*

	Hyperbilirubinemia group $n = 123$	Control group $n = 218$	p value
Male/female	73/50	130/88	NS*
Gestation age, wk	39.3 ± 0.8	39.4 ± 0.6	NS†
Birth weight, g	3230 ± 331	3276 ± 256	NS†

p values were given by χ^2 test* and Student's t test†.

Table 4. *Variations in UGT1A1 gene among newborn infants with hyperbilirubinemia and healthy controls*

UGT1A1 gene	Hyperbilirubinemia group $n = 123$	Control group $n = 218$	p value by χ^2 test
Wild type	46 (37.4%)	108 (49.5%)	0.032
Variation in promoter	13 (10.6%)	37 (17.0%)	NS
6/7	12	36	
A(TA)₇TAA/A(TA)₇TAA	1	1	
Variation at nucleotide 211	55 (44.7%)	56 (25.7%)	<0.001
211 G to A/normal	41	53	
211 G to A/211 G to A	14	3	
Variation at nucleotide 1091	1 (0.8%)	1 (0.5%)	NS
1091 C to T/normal	1	1	
Compound heterozygous variation	8 (6.5%)	16 (7.3%)	NS
6/7, 211 G to A/normal	5	6	
6/7, 686 C to A/normal	3	5	
6/7, 211 G to A/normal, 686 C to A/normal		2	
6/7, 1091 C to T/normal		2	
6/7, 211 G to A/normal, 1091 C to T/normal		1	

Table 5. *Comparison of various allele frequencies of UGT1A1 gene between neonates with hyperbilirubinemia and healthy controls*

Allele frequency	Newborns with hyperbilirubinemia	Healthy controls	p value
A (TA)₇TAA	0.089	0.124	NS*
211 G to A	0.301	0.156	<0.001*
686 C to A	0.012	0.018	NS†
1091 C to T	0.004	0.009	NS†

p values were given by χ^2 test* and χ^2 test with Yates' correction†.

shows the comparative severity of neonatal hyperbilirubinemia. The percentages with a bilirubin value $\geqq 342$ μM (20.0 mg/dL) and with persistent hyperbilirubinemia in the subjects carrying homozygous variation at nucleotide 211 were both significantly different from the neonates carrying wild type or other genotypes.

DISCUSSION

A variety of etiologic factors are involved in the development of neonatal hyperbilirubinemia (21). Among these risk factors, breast milk feeding probably is the predominant as that listed in Table 1. However, in some subjects, no known risk factors are identified and further etiologic investigations are required. The *UGT1A1* gene is considered as the first item requiring further investigation because UGT1A1 is the key enzyme regulating the elimination of bilirubin and the enzyme is labile and is not measured in neonates as it would involve liver biopsy. Variations in the promoter area of *UGT1A1* gene result in a reduced production of UGT1A1 (4, 5, 22) and variations within the coding region of this gene may result in dysfunction of UGT1A1 with abnormal structure (2, 3, 5, 22). These findings indicate that the variation of *UGT1A1* gene is possibly associated with neonatal hyperbilirubinemia. Kaplan *et al.* (10) and Cappellini *et al.* (13) found that the TA repeat

to A and 1091 C to T were not significantly different between these two groups.

Severity of neonatal hyperbilirubinemia. To understand whether the genotypes of *UGT1A1* gene influence the severity of hyperbilirubinemia, neonates of the study group carrying the variant nucleotide 211 were divided into two groups (heterozygous and homozygous variations) for further analysis. Table 6

Table 6. *Severity of neonatal hyperbilirubinemia in newborn infants with different UGT1A1 genotypes*

	Homozygous 211 G to A[a]	Wild type[b]	Other genotypes[c]	p value by χ^2 test*
Bilirubin $\geqq 342$ μmol/L	6/14	4/46	8/63	0.01[a vs b]
%	42.9	8.7	12.7	0.019[a vs c]
Persistent hyperbilirubinemia	8/14	7/46	5/63	0.005[a vs b]
%	57.1	15.2	7.9	<0.001[a vs c]

* With Yates' correction.

promoter polymorphism in *UGT1A1* gene increased the incidence of neonatal hyperbilirubinemia in G6PD deficiency. Iolascon *et al.* (12) reported that *UGT1A1* promoter polymorphism accounted for increased neonatal hyperbilirubinemia of hereditary spherocytosis. The studies of Bancroft *et al.* (11) and Monaghan *et al.* (14) showed that Gilbert's syndrome (homozygous A(TA)$_7$TAA variation) accelerated development of hyperbilirubinemia and contributed to prolonged hyperbilirubinemia in newborn infants. Akaba *et al.* (15) and Maruo *et al.* (16, 23) described that the allele frequency of the Gly71Arg variation in *UGT1A1* gene was significantly higher in neonates with hyperbilirubinemia than in healthy adult controls and neonates without hyperbilirubinemia. The results of our study confirm this finding. Therefore, neonates having the variant genotype at nucleotide 211 may be at risk for the development of neonatal hyperbilirubinemia. Interestingly, the 211 G to A variation (Gly71Arg) has not been reported in Caucasians (4). On the other hand, the frequencies of the variant promoter in Taiwan Chinese neonates (Table 4) and adults (17) were quite low (0.089–0.143) compared with Caucasians (0.357–0.415) (4, 24–26). In this study, the allele frequency of A(TA)$_7$TAA in neonates with hyperbilirubinemia who had no known risk factors was not different from that of healthy controls. Thus, the variant site associated with neonatal hyperbilirubinemia in our study subjects was at nucleotide 211 rather than in the promoter area, unlike previous findings in Caucasians (10–14). These ethnic differences may also be observed in Gilbert's syndrome and deserve further investigation.

In our previous study, we found that the bilirubin levels were correlated well to the frequencies of variant *UGT1A1* gene (17). Although the variation of Gly71Arg in *UGT1A1* gene was found to be associated with hyperbilirubinemia in Japanese neonates (15, 16, 23), the comparisons of disease severity among those with different UGT1A1 genotypes were not analyzed because the sample size was small. In this study, among the hyperbilirubinemic newborn infants without known risk factors, those who carried homozygous 211 G to A variation had worse outcome (Table 6). Moreover, the peak bilirubin level was 416 μM (24.3 mg/dL) at day 10 in a neonate carrying the homozygous 211 variant. This neonate had the most severe disease among the 123 subjects of the study group. Two units (500 mL) of blood exchange treatment were given to prevent kernicterus. In the in vitro expression study by Yamamoto *et al.* (27), the 211 G to A variation in the homozygous and heterozygous state decreased the UGT1A1 enzyme activities to 32.2% and 60.2% of normal, respectively. The decreased enzyme activities are thought to cause moderate and mild delayed elimination of bilirubin (5). It is not surprising that the clinical symptoms in the neonates who carried homozygous

variation at nucleotide 211 were more severe than in those who carried heterozygous 211 variant. In this study, as that we previously found in healthy Taiwan Chinese adults (17), some neonates carried compound heterozygous variations in *UGT1A1* gene. The 686 C to A variation was associated with 6/7 variant, and this correlation warrants further investigation. A few subjects carried the 1091 C to T variation. Such a variation has not been reported in ethnic groups other than Taiwan Chinese.

Other genetic factors, such as G6PD deficiency, combined with the variation in *UGT1A1* gene may influence the bilirubin levels in neonates (10, 13). Our previous research indicated that most (78.3%) of the G6PD deficient neonates who subsequently suffered from neonatal hyperbilirubinemia carried the nucleotide 1376 G to T mutation in *G6PD* gene (19). In this report, the data of risk factors indicated that the incidence of G6PD deficiency in the neonates with hyperbilirubinemia was 4.8% (15/314) (Table 1), which was approximately double that in the general population of Taiwan Chinese (19). However, the interaction of G6PD deficiency and variation in *UGT1A1* gene in neonatal hyperbilirubinemia in Asian newborn infants remains to be determined. Such a study was performed by us very recently and will be published (28).

CONCLUSION

In conclusion, the 211 G to A variation of *UGT1A1* gene is a risk factor for neonatal hyperbilirubinemia. The high incidence of neonatal hyperbilirubinemia in Taiwan Chinese is partly attributable to the high frequency of this variation. The test for this variation will be useful to investigate the causes of neonatal hyperbilirubinemia without known risk factors. Pediatricians should closely follow hyperbilirubinemic newborn infants who carry homozygous 211 G to A variation in *UGT1A1* gene.

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