211 G to A Variation of UDP-Glucuronosyl Transferase 1A1 Gene and Neonatal Breastfeeding Jaundice

HUNG-CHIEH CHOU, MEI-HUEI CHEN, HWAI-I YANG, YI-NING SU, WU-SHIUN HSIEH, CHIEN-YI CHEN, HUEY-LING CHEN, MEI-HWEI CHANG, AND PO-NIEN TSAO

Departments of Pediatrics and Medical Genetics [H.-C.C., W.-S.H., C.-Y.C., H.-L.C., M.-H.Cha., P.-N.T.], Graduate Institute of Clinical Medicine, and Graduate Institute of Clinical Genomics [Y.-N.S.], National Taiwan University Hospital, Taipei 100, Taiwan; Department of Pediatrics [M.-H.Che.], Cardinal Tien Hospital Yung Ho Branch, Taipei 231, Taiwan; Genomics Research Center [H.-I.Y.], Academia Sinica, Taipei 115, Taiwan

ABSTRACT: Breastfeeding jaundice is a common problem in neonates who were exclusively breastfed, but its pathogenesis is still unclear. The uridine diphosphate glucuronosyl transferase 1A1 (*UGT1A1*) gene polymorphism was shown to contribute to the development of neonatal hyperbilirubinemia. We hypothesize that the variation of *UGT1A1* gene may contribute to neonatal breastfeeding jaundice. We prospectively enrolled 688 near-term and term infants who were exclusively breastfed (BF group) or were supplemented by infant formula partially (SF group) before onset of hyperbilirubinemia. Genotyping of the promoter and exon1 of *UGT1A1* was performed in all neonates. Neonates in BF group had a significantly higher maximal body weight loss ratio, peak bilirubin level, and a greater incidence of hyperbilirubinemia than those in SF group. Neonates with nucleotide 211 GA or AA variation in *UGT1A1* genotypes had higher peak serum bilirubin levels and higher incidence of hyperbilirubinemia than WT (GG). This phenomenon was only seen in BF group but not in SF group when subset analysis was performed. This suggests that neonates who carry the nucleotide 211 GA or AA variation within coding region in *UGT1A1* gene are more susceptible to develop early-onset neonatal breastfeeding jaundice. (*Pediatr Res* 69: 170–174, 2011)

Neonatal hyperbilirubinemia is a common problem and is of concern for both pediatricians and parents. The incidence and severity of neonatal hyperbilirubinemia are different among races. The peak serum levels of unconjugated bilirubin in full-term Asian (Japanese, Korean, or Chinese) and American Indian neonates are almost double as those in Caucasian and black populations (1,2). The incidence of kernicterus is also higher among Asian newborn infants (3). These findings suggest that genetic factors are involved in the development of neonatal hyperbilirubinemia.

Uridine diphosphate glucuronosyl transferase 1A1 (*UGT1A1*) is the key enzyme for bilirubin conjugation, and mutations of *UGT1A1* cause unconjugated hyperbilirubinemia syndromes known as Crigler-Najjar syndrome and Gilbert's syndrome (4–6). Recently, homozygous A(TA)₇TAA variation in promoter region of *UGT1A1* gene was found to be associated with neonatal hyperbilirubinemia in western people (7–14). However, in Japa-

nese, Koreans, and Taiwanese studies, the high allele-frequency of Gly71Arg, but not promoter polymorphism, in *UGT1A1* gene was found to be responsible for neonatal hyperbilirubinemia (15–19). These reports reveal that the relationship between the site of variant *UGT1A1* gene and neonatal hyperbilirubinemia may differ among races.

Breastfeeding has been shown to have several advantages for infants, mothers, and families (20,21). However, there is a strong association between breastfeeding and an increase in the risk of early neonatal hyperbilirubinemia and severe hyperbilirubinemia (22–25). Decreased caloric intake, inhibition of hepatic excretion of bilirubin, and an increase in intestinal absorption of bilirubin (enterohepatic circulation) are suggested mechanisms for early neonatal hyperbilirubinemia associated with breastfeeding (24). Recently, a large population study showed the important role of fasting, but not breastfeeding *per se*, in the pathogenesis of neonatal hyperbilirubinemia (26). In addition, Ishihara *et al.* (27) demonstrated that mutation in the TATA box or in the coding region of *UGT1A1* was found to have significantly higher increment of serum bilirubin values in healthy adult subjects than individuals without any mutation after caloric restriction. We therefore investigated whether there were interactions between genetic polymorphism of *UGT1A1* gene and breastfeeding itself, or the fasting effect, affecting circulating bilirubin levels in the first week of life.

METHODS

Subjects. We prospectively collected cord blood of neonates with GA 35 wk or older, born between April 2002 and November 2004 in the department of Pediatrics of National Taiwan University Hospital, Taipei, Taiwan. Medical records including GA, birth weight (BW), body weight loss [(birth body weight – daily body weight)/birth body weight × 100%], total serum bilirubin levels, and peak bilirubin levels before phototherapy were reviewed. The serum bilirubin levels were routinely measured in all the neonates at 3 d old or when the icteric skin was found. The median onset age of hyperbilirubinemia was 3 d old. Those with risk factors for developing neonatal hyperbilirubinemia, such as evidence of hemolysis (positive Coombs' test), glucose-6-phosphate dehydrogenase (G6PD) deficiency, cephalohematoma, congenital infection, perinatal asphyxia, and major organ anomaly were excluded. Hyperbilirubinemia and the criteria of phototherapy were defined

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Correspondence: Po-Nien Tsao, M.D., Ph.D., Department of Pediatrics, National Taiwan University Hospital, No. 7, Chung Shan South Road, Taipei 100, Taiwan; e-mail: tsao pn@ntu.edu.tw

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Abbreviations: BF, breastfeeding; BW, birth weight; G6PD, glucose-6-phosphate dehydrogenase; SF, supplementary feeding; UGT1A1, uridine diphosphate glucuronosyl transferase 1A1

according to the 2004 American Academy of Pediatrics guideline for phototherapy (28), except that we started phototherapy for all infants whose serum bilirubin levels were >15 mg/dL. The Human Research Ethical Committee of National Taiwan University Hospital approved the study, and informed consent was obtained from the parents before enrollment of the study.

Breastfeeding (BF) was defined as infants who were exclusively breastfed without supplementation of formula at any time or before developing hyperbilirubinemia. Supplementary feeding (SF) was defined as infants who were breastfed and received additional formula supplements. The exclusive infant formula feeding was excluded. In our nursery, breastfeeding is encouraged but supplementary formula will be given because of inadequate breastfeeding as judged by parent and clinician or maternal preference. However, supplementary formula is routinely used if body weight loss after birth was significant ($\geq 10\%$).

UGT1A1 genotyping. The genotyping for the promoter region and exon 1 of UGT1A1 was performed as previous described by a technician who was blind to the grouping of the subjects (29). Briefly, total genomic DNA was isolated from cord blood using a Puregene DNA isolation kit (Gentra System, Inc., Minneapolis, MN) according to the manufacturer's instructions. PCR was performed in a 25- μ L reaction volume containing 100 ng of genomic DNA template as provided by the manufacturer. Amplification was performed in a multiblock system thermocycler (ThermoHybaid, Ashford, United Kingdom): initial denaturation step at 95°C for 10 min, followed by 23 cycles consisting of denaturation at 94°C for 30 s, annealing at 56°C for 60 s, extension at 72°C for 30 s, and then a final extension step at 72°C for 10 min.

DHPLC software, using Wave DNA Fragment Analysis System (Transgenomic Inc., San Jose, CA), recorded peak heights corresponding to the signal from each PCR product. The temperature for optimal heteroduplex analysis was determined using either the DNA melt software described by Jones *et al.* (30) or by WAVEMaker software (Transgenomic). Running conditions for dosage analysis are adjusted and modified by each setting of PCR products.

Sequencing reactions were performed for all samples by using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Inc., Foster City, CA). Electrophoresis was carried out using a Genetic Analyzer 310 (PE Applied Biosystems, Inc.) equipped with long-read sequencing capillary and POP-6 sequencing polymer (PE Applied Biosystems, Inc.). Primers used for direct sequencing reactions were the same as PCR reaction.

Statistical analysis. SPSS 13.0 for Windows software program (SPSS Inc., Chicago, IL) was used for statistical analysis. Differences in distribution of several categorical and continuous variables between BF and SF groups were analyzed using χ^2 test, Fisher's exact test, and *t* test where appropriate. The simple linear regression and the Cochran-Armitage trend test were used to assess whether the peak serum bilirubin level and the incidence of hyperbilirubinemia increased with numbers of non-WT alleles in genotypes, respectively. Multivariate-adjusted ORs and 95% CIs of hyperbilirubinemia were estimated for genetic polymorphisms of UGT1A1 gene and various risk factors selected by univariate analysis by using multiple logistic regression analysis. Subset analysis was further carried out to evaluate the potential synergistic effect between coding region 211 of the UGT1A1 gene and stratifying factors including group, sex of baby, as well as the GA. Test of interaction was performed by adding a product term in the model. All tests were two-sided. The *p* value <0.05 was considered statistically significant.

RESULTS

Clinical data. After excluding 57 babies (12 cases had evidence of hemolysis, 16 cases had G6PD deficiency, 20 cases had cephalohematoma, and nine cases had major organ abnormality), a total of 688 newborn infants were enrolled in analyses. The characteristics of eligible population are listed in Table 1. Among these, 180 (26.2%) infants who had hyperbilirubinemia required phototherapy. The mean GA, BW, sex, and the rate of cesarean section were not significantly different between BF and SF groups. However, the neonates in BF group had a significantly higher maximal body weight loss ratio, peak bilirubin level, and a greater incidence of hyperbilirubinemia than those in SF group.

UGT1A1 genotypes. By UGT1A1 genotyping, WT was found in 354 of 688 infants (51.5%) for both coding region 211 and promoter region. The incidences of heterozygous

Table 1. Dermographic characteristics of the 688 infants enrolled in this study*

Characteristics	BF group	SF group	<i>p</i>
<i>N</i>	341	347	
Male	163 (47.8)	182 (52.4)	0.223
Cesarean delivery	93 (27.3)	105 (30.3)	0.420
GA (wk), mean (SD)	38.9 (1.2)	38.8 (1.3)	0.266
BW (g), mean (SD)	3128 (351)	3214 (464)	0.911
Maximal body weight loss (%), mean (SD)†	8.5 (2.2)	6.3 (2.2)	<0.001
Peak bilirubin level (mg/dL), mean (SD)	12.4 (3.0)	11.6 (3.2)	<0.001
Hyperbilirubinemia	103 (30.2)	77 (22.2)	0.017

* Data are expressed as *n* (%) unless otherwise noted.

† Maximal body weight loss was calculated as maximal body weight loss in comparison to BW divided by birth body weight.

Table 2. Results for UGT1A1 genetic polymorphisms in BF and SF groups*

UGT1A1 genotypes	BF group (<i>N</i> = 341)	SF group (<i>N</i> = 347)	<i>p</i>
Coding region 211			0.44
WT	234 (68.6)	244 (70.3)	
G/A	102 (29.9)	94 (27.1)	
A/A	5 (1.5)	9 (2.6)	
Promoter			0.13
WT	265 (77.7)	274 (79.0)	
A(TA) ₆ TAA/A(TA) ₇ TAA (6/7)	72 (21.1)	62 (17.9)	
A(TA) ₇ TAA/A(TA) ₇ TAA (7/7)	4 (1.2)	11 (3.2)	

* Data are expressed as *N* (%).

variation within coding region (211 G to A/G), heterozygous variation within promoter A(TA)₆TAA/A(TA)₇TAA (6/7), homozygous variation with either 211A/A or A(TA)₇TAA/A(TA)₇TAA (7/7) were 28.5, 19.5, 2, and 2.2%, respectively. Among them, 24 (3.5%) cases were compound heterozygous variation (promoter 6/7 and 211 G to A/normal). As shown in Table 2, there was no significant difference in frequencies of genetic polymorphisms of UGT1A1 genotypes between BF and SF groups.

Neonates carrying the 211 G to A variation of UGT1A1 gene were susceptible to breastfeeding jaundice. In general, the peak bilirubin levels before phototherapy were higher in BF than SF group when we analyzed the neonates according to their UGT1A1 genotyping (Table 3). However, this difference was only significant for the subgroups that carried the WT and heterozygous variants on coding region 211 and the WT variant on promoter region of UGT1A1 gene (Table 3). Similarly, the incidence of hyperbilirubinemia was also higher in BF groups than SF groups, especially with statistical significance among subjects who carried G/A variant on coding region 211 and WT variant on promoter region of UGT1A1 gene. In addition, a statistically significant dose effect for 211 G to A variation of UGT1A1 gene was found on both the peak bilirubin level (*p* for trend test = 0.004) and the incidence of hyperbilirubinemia among exclusive breastfeeding babies (*p* for trend test = 0.001; Table 3), although there was no statistical significance in homozygous variants on coding 211 (e.g. A/A), which may be a result of small sample size. In contrast, promoter TATA box variation has no such effect.

Table 3. Correlation of the clinical data for the breast feeding versus supplementary feeding newborns with different UGT1A1 genotypes

UGT1A1 genotypes (case number)	Peak serum bilirubin level			Incidence of hyperbilirubinemia		
	BF, mean $\pm$ SD	SF, mean $\pm$ SD	<i>p</i>	BF, <i>n</i> (%)	SF, <i>n</i> (%)	<i>p</i>
Coding region 211						
WT (478)	12.2 $\pm$ 2.8	11.4 $\pm$ 3.2	0.006	59/234 (25.2)	52/244 (21.3)	0.32
G/A (196)	13.0 $\pm$ 3.3	12.0 $\pm$ 3.0	0.019	40/102 (39.2)	21/94 (22.3)	0.011
A/A (14)	14.5 $\pm$ 2.5	12.3 $\pm$ 3.0	0.19	4/5 (80.0)	4/9 (44.4)	0.20
<i>p</i> for trend test	0.004	0.10		0.001	0.30	
Promoter						
WT (539)	12.6 $\pm$ 3.1	11.7 $\pm$ 3.1	<0.001	83/265 (31.4)	64/274 (23.4)	0.038
A(TA) ₆ TAA/A(TA) ₇ TAA (6/7) (134)	12.0 $\pm$ 2.6	11.0 $\pm$ 3.6	0.05	19/72 (26.8)	8/62 (12.9)	0.052
A(TA) ₇ TAA/A(TA) ₇ TAA(7/7)(15)	9.8 $\pm$ 4.8	12.6 $\pm$ 2.5	0.15	1/4 (25.0)	5/11 (45.5)	0.47
<i>p</i> for trend test	0.040	0.60		0.43	0.87	

Table 4. Multivariate regression analysis of early onset neonatal hyperbilirubinemia

Variable	Adjusted OR (95% CI)	<i>p</i>
Sex of baby		
Female	Referent	
Male	0.91 (0.63–1.31)	0.61
GA (wk)	0.82 (0.70–0.97)	0.017
Cesarean delivery		
No	Referent	
Yes	0.90 (0.60–1.35)	0.61
BW (kg)	1.04 (0.63–1.71)	0.88
Maximal body weight loss (%)	1.11 (1.02–1.21)	0.016
Group		
SF	Referent	
BF	1.28 (0.86–1.91)	0.22
UGT1A1 gene coding region 211		
WT	Referent	
G/A	1.47 (1.00–2.15)	0.048
A/A	4.12 (1.33–12.76)	0.014
UGT1A1 gene promoter		
WT	Referent	
A(TA) ₆ TAA/A(TA) ₇ TAA (6/7)	0.74 (0.46–1.20)	0.22
A(TA) ₇ TAA/A(TA) ₇ TAA(7/7)	2.29 (0.77–6.81)	0.14

Neonates who were exclusively breastfed and carried homozygous variant (A/A) on coding region 211 of *UGT1A1* gene had highest mean level of peak serum bilirubin (14.5 mg/dL) as well as the highest incidence of hyperbilirubinemia (80%).

211 G to A variation of UGT1A1 gene was an independent risk factor of early neonatal hyperbilirubinemia. Multivariate regression analysis for neonatal hyperbilirubinemia showed that neonates who have lower GA, higher maximal body weight loss, or UGT1A1 211 G to A variations were risk predictors of development of neonatal hyperbilirubinemia. The 211 G to A genotypes of *UGT1A1* gene was the most important independent risk factor of neonatal hyperbilirubinemia with adjusted OR (95% CI) of 1.48 (1.01–2.16) and 4.14 (1.34–12.82), for G/A and A/A variants, respectively, compared with the WT variants (Table 4).

Subgroup analysis of genetic polymorphism of coding region 211 of UGT1A1 gene on risk of neonatal hyperbilirubinemia. As shown in Table 5, the association between genotype of *UGT1A1* gene coding region 211 and risk of hyperbilirubinemia remained consistent after stratification according to feeding group, maximal body weight loss, as well as sex and GA of baby. Interestingly, after adjustment for BW,

Table 5. Subgroup analysis of UGT1A1 gene coding region 211 genotype and risk of hyperbilirubinemia

Stratifying variable	UGT1A1 gene coding region 211, Adjusted OR (95% CI)*		
	WT	G/A	A/A
Group			
SF	Referent	1.13 (0.61–2.12)	2.51 (0.60–10.47)
BF	Referent	2.10 (1.26–3.50)	14.79 (1.61–135.32)
Sex of baby			
Female	Referent	2.15 (1.24–3.74)	4.50 (1.07–18.93)
Male	Referent	1.27 (0.73–2.21)	3.92 (0.77–20.04)
GA (wk)			
$\leq 39^{\dagger}$	Referent	1.75 (1.11–2.77)	4.54 (1.33–15.47)
> 39	Referent	1.33 (0.62–2.83)	2.82 (0.25–32.49)
Maximal body weight loss (%)			
≤ 7.33	Referent	1.33 (0.75–2.35)	2.83 (0.46–17.52)
> 7.33	Referent	1.63 (0.99–2.67)	5.41 (1.31–22.35)

* Adjustment for birth weight.

$\dagger$ Mean value.

neonates carrying G/A and A/A variants had substantial high risk of hyperbilirubinemia than the WT in BF group rather than in SF group; as well as in neonates with maximal body weight loss $> 7.33\%$ rather than in those with maximal body weight loss $\leq 7.33\%$. Neonates who carried homozygous 211 G to A variation of *UGT1A1* gene and were exclusively fed with breast milk had highest relative risk (OR, 11.72; 95% CI, 1.28–106.98) to develop neonatal hyperbilirubinemia in BF group; whereas the corresponding OR (95% CI) was 3.10 (0.80–12.02) in SF group. Similarly, the corresponding OR (95% CI) was 5.39 (1.30–22.25) and 2.83 (0.46–17.52), respectively, in groups with maximal body weight loss $> 7.33\%$ and $\leq 7.33\%$.

DISCUSSION

The etiology of breastfeeding jaundice is unclear. Decreased caloric intake resulting in increased absorption of intestine bilirubin due to poor breast milk intake is the suggested mechanism (24,31). Recently, Bertini *et al.* (26) also demonstrated that the data confirm the important role of fasting in the pathogenesis of early onset neonatal hyperbilirubinemia. However, not all breastfed neonates develop breastfeeding jaundice. This suggests that there are some contributory factors that may interact with breastfeeding re-

sulting in neonatal hyperbilirubinemia. In this study, we demonstrate that UGT1A1 211 G to A variation is an important contributor of breastfeeding jaundice.

The *UGT1A1* gene variation is well known to be associated with neonatal hyperbilirubinemia (8,10–19,32,33). Gene-gene interaction between *UGT1A1* and G6PD has been shown to contribute to the pathogenesis of neonatal hyperbilirubinemia (9,10,16). In addition, we and Maruo *et al.* (33,34) demonstrated that defects of *UGT1A1* gene are an underlining cause of prolonged unconjugated hyperbilirubinemia associated with breast milk (34). These data suggest that *UGT1A1* gene variation may also interact with neonatal environment, breast milk for example, and contributes to neonatal hyperbilirubinemia. Therefore, we speculate that the polymorphism of *UGT1A1* gene may also contribute to breastfeeding-related early neonatal hyperbilirubinemia. The results of this study support our speculation.

Interestingly, the positive association of 211 G to A variation of *UGT1A1* gene on neonatal hyperbilirubinemia was observed predominantly in BF group rather than in SF group. This result implies that there seems to be a gene-environment interaction between *UGT1A1* gene coding region 211 variation and breastfeeding. This gene-environment interaction may explain, at least in part, why some neonates were more susceptible to breastfeeding jaundice. Previously, Huang *et al.* (17) showed that variation at nucleotide 211 of *UGT1A1* gene is a risk factor for development of neonatal hyperbilirubinemia in neonates who were not fed with breast milk. In this study, we clearly demonstrated that heterozygous 211 G to A is not significantly associated with neonatal hyperbilirubinemia in neonates who were not exclusively breastfed. The discrepancy between these two studies may be due to the difference in study population. Previously, many neonates were fed with infant formula or mixed with breast milk in Taiwan, and the incidence of exclusive breastfeeding was low. As breastfeeding promotion program was popularized recently in Taiwan, most of the neonates were fed with breast milk, with or without minimal supplement of infant formula in the first few days of life (35). Although SF groups in our study and in study by Huang population were not exclusive breastfeeding group, the SF group in this study included only those neonates who were breastfeeding but received additional formula supplements partially. By contrast, the neonates in the report by Huang included those fed with infant formula exclusively without breastfeeding, which were totally excluded from our study population.

The mechanism of *UGT1A1* gene variations and breastfeeding interaction resulting in early onset neonatal hyperbilirubinemia (breastfeeding jaundice) is not clear. Previous studies have shown that fasting is an important factor in the pathogenesis of breastfeeding jaundice (24,26,31). Ishihara *et al.* (27,36) clearly demonstrated that human subjects with *UGT1A1* mutations showed higher levels of increment of serum bilirubin after caloric restriction than individuals without mutation. These data suggest that neonates with *UGT1A1* gene variations are more susceptible for fasting hyperbilirubinemia resulting from breastfeeding than those with WT. In the present study, dose-response relationship of 211 G to A

variation of *UGT1A1* gene on peak bilirubin level and incidence of breastfeeding jaundice was noted. The result from the subgroup analysis on maximal body weight loss as shown in Table 5 supports the above-mentioned hypothesis as well. In conclusion, the nucleotide 211 variation in *UGT1A1* gene coding region is an independent risk factor for developing breastfeeding jaundice. This association was predominantly seen in exclusive breastfeeding babies with high maximal body weight loss. It implicates the possible mechanism that may be associated with early exclusive breastfeeding-induced fasting, therefore resulting in significant increment of bilirubin levels in neonates who carry the nucleotide 211 variation in *UGT1A1* gene. This data may explain, at least partially, why some neonates with exclusive breastfeeding are more susceptible to develop early-onset neonatal breastfeeding jaundice than others.

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