ORIGINAL ARTICLE

Association of breast-fed neonatal hyperbilirubinemia with *UGT1A1* polymorphisms: 211G > A (G71R) mutation becomes a risk factor under inadequate feeding

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Breastfeeding jaundice is a well-known phenomenon, but its pathogenesis is still unclear. Increased production of bilirubin, impaired hepatic uptake and metabolism of bilirubin, and increased enterohepatic circulation of bilirubin account for most cases of pathological neonatal hyperbilirubinemia. We previously reported that 211G > A (G71R) mutation of the *UGT1A1* gene is prevalent in East Asians and is associated with the development of neonatal hyperbilirubinemia. Recently, significant association of G71R mutation with hyperbilirubinemia in breast-fed neonates was reported. We enrolled 401 full-term Japanese infants, who were exclusively breast-fed without supplementation of formula before developing hyperbilirubinemia, and classified them into two groups based on the degree of maximal body weight loss during the neonatal period. We analyzed the sex, gestational age, delivery mode, body weight at birth, maximal body weight loss and genotypes of G71R and (TA)₇ polymorphic mutations of *UGT1A1*. Statistical analysis revealed that maximal body weight loss during the neonatal period is the only independent risk factor for the development of neonatal hyperbilirubinemia. The effect of G71R mutation on neonatal hyperbilirubinemia is significant in neonates with 5% or greater maximal body weight loss and its influence increases in parallel with the degree of maximal body weight loss. Our study indicates that G71R mutation is a risk factor for neonatal hyperbilirubinemia only in infants with inadequate breastfeeding and suggests that adequate breastfeeding may overcome the genetic predisposing factor, G71R mutation, for the development of neonatal hyperbilirubinemia.

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INTRODUCTION

Unconjugated hyperbilirubinemia in neonates is a physiological and common phenomenon. Bilirubin production is increased in the neonate because of the larger erythrocyte volume, shortened erythrocyte lifespan, heme and heme precursors degraded from the fetal extramedullary hematopoietic tissue, and, possibly, increased turnover of cytochromes.¹ The ability to conjugate bilirubin is also extremely low in neonates at term, being about 1% of adult values.² Neonatal hyperbilirubinemia is also probably associated with other factors such as an immaturity of hepatic bilirubin uptake and intracellular bilirubin transport, and the increased enterohepatic circulation of bilirubin. The imbalance between the production and elimination of bilirubin leads to varying levels of hyperbilirubinemia in every neonate. The propensity toward neonatal hyperbilirubinemia in East Asians is well known.¹ Association of mutation of the *UGT1A1* gene,

(TA)₇ causing Gilbert syndrome, has been reported.³ In 1998, we found that 211G > A (G71R) mutation of the *UGT1A1* gene is very common among Japanese, Koreans and Chinese, and this mutation is associated with neonatal hyperbilirubinemia.^{4,5} Recently, the association of G71R mutation with neonatal hyperbilirubinemia was also confirmed in Asians⁶ and was especially significant in breast-fed neonates.^{7,8} Regarding breastfeeding jaundice, appropriately known as breast-non-feeding jaundice, it has been considered to result from inadequate calorie intake.⁹

In the present paper, we studied the association of G71R and (TA)₇ mutations and hyperbilirubinemia in breast-fed Japanese neonates, and found that the maximal body weight loss during the neonatal period is an independent risk factor for the development of neonatal hyperbilirubinemia, and G71R mutation is a significant risk factor only in neonates with 5.0% or greater maximal body weight loss.

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SUBJECTS AND METHODS

Subjects

The subjects were recruited from among the Japanese full-term and breast-fed neonates born at Yamagata Prefectural Central Hospital from July 1, 2008 to June 30, 2010. We excluded neonates who had factors that would affect the level of serum bilirubin, such as hemolytic anemia, neonatal asphyxia, maternal diabetes, congenital heart or intestine malformation, infections and parenteral fluid therapies. We finally studied the 401 neonates who were exclusively breast-fed without supplementation of formula before developing hyperbilirubinemia. Main characteristics of the 401 neonates were as follows: male-to-female ratio, 221:180, gestational age, 39.5 ± 1.2 (mean $\pm$ s.d.) weeks; and birth weight, 3080 ± 325 (mean $\pm$ s.d.) group. Neonates with severe hyperbilirubinemia were treated with phototherapy according to the criteria. Medical records including gestational age, birth weight, body weight loss [(birth body weight – daily body weight)/birth body weight $\times 100\%$], total serum bilirubin levels and peak bilirubin levels before phototherapy were reviewed. The Ethics Committee of the Yamagata University School of Medicine approved this study. After informed consent was obtained from the parents, genomic DNA was isolated from spare dried blood spots from neonatal screening cards, as described previously.¹⁰

The 401 neonates were classified into group A (neonates showing <10% body weight loss during the neonatal period) and group B (neonates showing 10% or greater body weight loss during the neonatal period) (Table 1).

Assessment of serum bilirubin level and criteria for phototherapy

Serum bilirubin of the neonates was assessed with a Jaundice Meter (model 103; Minolta, Osaka, Japan) once a day, at the same time, during hospitalization or at least during the first week of life. When the reading of the transcutaneous bilirubinometer reached the criterion for further evaluation, the total serum bilirubin concentration was measured. Phototherapy was initiated if the measured bilirubin level exceeded the criterion as follows: $171 \mu\text{mol l}^{-1}$ at day 1, $239 \mu\text{mol l}^{-1}$ at day 2, $274 \mu\text{mol l}^{-1}$ at day 3, $291 \mu\text{mol l}^{-1}$ at day 4, $308 \mu\text{mol l}^{-1}$ at day 5 and $342 \mu\text{mol l}^{-1}$ at day 6.

UGT1A1 gene analysis

The (TA)₇ mutation in the promoter and G71R mutation in exon 1 of the *UGT1A1* gene were analyzed by fragment analysis¹¹ and TaqMan Drug Metabolism Genotyping Assay (c_559715_20) (PE Applied Biosystems, Foster City, CA, USA), respectively.

Statistical analysis

R statistical package 2.11.1 for Windows was used for statistical analysis. Differences in the distribution of several categorical and continuous variables between A and B groups were analyzed using the χ^2 -test and Mann–Whitney *U*-test where appropriate. Simple linear regression and the Cochran–Armitage trend test were used to assess whether the peak serum bilirubin level and incidence of hyperbilirubinemia increased with numbers of non wild-type alleles in genotypes, respectively. Multivariate-adjusted odds ratios (ORs) and

95% confidence intervals (CIs) of hyperbilirubinemia were estimated for genetic polymorphisms of the *UGT1A1* gene and various risk factors selected by univariate analysis using multiple logistic regression analysis. Subset analysis was carried out to evaluate the potential synergistic effect between G71R mutation of the *UGT1A1* gene and various levels of maximal body weight loss. A test of interaction was performed by adding a product term to the model. All tests were two-sided. A *P*-value <0.05 was considered significant.

RESULTS

A total of 401 neonates were enrolled in the study, and were classified into two groups. Groups A and B included neonates showing a less than 10% body weight loss and 10% or greater body weight loss during the neonatal period, respectively. As shown in Table 1, 56 neonates (14%) showed hyperbilirubinemia and required phototherapy. The neonates of group B had a significantly higher peak bilirubin level and incidence of hyperbilirubinemia, higher frequency of cesarean delivery, and shorter gestational period. Sex and body weight at birth were not significantly different between the two groups.

UGT1A1 genotyping of the 401 neonates showed that gene frequencies of G71R and (TA)₇ mutations were 0.18 and 0.13, respectively. As shown in Table 2, the numbers of neonates carrying the (TA)₇ mutation were not significantly different between the two groups, but neonates carrying the G71R mutation were more frequently detected in group A.

The peak bilirubin level and incidence of hyperbilirubinemia were higher in the neonates of group B compared with those of group A (Table 1). However, significant increases in the peak bilirubin level and incidence of hyperbilirubinemia were only noted in the neonates carrying heterozygous G71R mutation or homozygous (TA)₆ (wild-type) of the promoter of group B (Table 3). The gene dose effect for G71R mutation was not confirmed, possibly because of the small sample size to carry out statistical tests of the neonates with homozygous G71R mutation. (TA)₇ mutation had no increasing effect on the peak bilirubin level and incidence of hyperbilirubinemia.

Multivariate regression analysis for neonatal hyperbilirubinemia revealed that only maximal body weight loss was an independent risk factor for the development of neonatal hyperbilirubinemia with adjusted OR (95% CI) of 1.25 (1.11–1.41) (Table 4). The G71R mutation was not a significant independent risk factor for neonatal hyperbilirubinemia. On the other hand, heterozygous (TA)₇ mutation and cesarean delivery rather significantly decreased the risk of neonatal hyperbilirubinemia.

Maximal body weight loss was the only independent risk factor for the development of neonatal hyperbilirubinemia, and the association

Table 1 Demographic characteristics of the 401 infants enrolled in this study^a

Characteristics	Group A	Group B	P
N	299	102	
Male	169 (56.5)	52 (51.0)	0.331
Cesarean delivery	43 (14.4)	27 (26.5)	0.005
GA (weeks), mean $\pm$ s.d.	39.7 ± 1.2	39.2 ± 1.3	0.005
BW (g), mean $\pm$ s.d.	3082 ± 318	3076 ± 351	0.573
Maximal body weight loss (%), mean $\pm$ s.d. ^b	7.2 ± 1.8	11.8 ± 1.3	<0.001
Peak bilirubin level (mg dl ⁻¹), mean $\pm$ s.d.	11.9 ± 2.5	12.4 ± 3.0	0.046
Hyperbilirubinemia	35 (11.7)	21 (20.6)	0.025

Abbreviations: BW, birth weight; GA, gestational age.

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

^bMaximal body weight loss was calculated as maximal body weight loss in comparison with body weight divided by birth body weight.

Table 2 Results of *UGT1A1* genetic polymorphisms in A and B groups^a

<i>UGT1A1</i> genotypes	Group A (N = 299)	Group B (N = 102)	P
<i>Coding region 211</i>			
G/G	188 (62.9)	79 (77.5)	0.026
G/A	105 (35.1)	22 (21.6)	
A/A	6 (2.0)	1 (0.9)	
<i>Promoter</i>			
(TA) ₆ /(TA) ₆	234 (78.3)	69 (67.6)	0.087
(TA) ₆ /(TA) ₇	59 (19.7)	29 (28.4)	
(TA) ₇ /(TA) ₇	6 (2.0)	4 (3.9)	

^aData are expressed as *N* (%).

Table 3 Correlations of the peak serum bilirubin level and incidence of hyperbilirubinemia in group A versus group B with different *UGT1A1* genotypes

<i>UGT1A1</i> genotypes (case number)	Peak serum bilirubin level			Incidence of hyperbilirubinemia		
	A, mean $\pm$ s.d.	B, mean $\pm$ s.d.	P	A, n (%)	B, n (%)	P
<i>Coding region 211</i>						
G/G (267)	11.7 $\pm$ 2.5	11.8 $\pm$ 3.1	0.371	21/188 (11.2)	10/79 (12.7)	0.729
G/A (127)	12.2 $\pm$ 2.6	14.1 $\pm$ 2.0	0.001	13/105 (12.4)	11/22 (50.0)	4.2×10^{-5}
A/A (7)	12.9 $\pm$ 2.4	—	—	1/6 (16.7)	0/1 (0)	—
P for trend test	0.067	0.002		0.657	<0.001	
<i>Promoter</i>						
(TA) ₆ /(TA) ₆ (303)	12.1 $\pm$ 2.4	12.8 $\pm$ 3.2	0.009	29/234 (12.4)	20/69 (29.0)	0.001
(TA) ₆ /(TA) ₇ (88)	11.2 $\pm$ 2.5	11.6 $\pm$ 2.5	0.394	6/59 (10.2)	1/29 (3.4)	0.273
(TA) ₇ /(TA) ₇ (10)	11.6 $\pm$ 3.7	10.4 $\pm$ 2.3	0.454	0/6 (0)	0/4 (0)	—
P for trend test	0.021	0.027		0.376	0.003	

Table 4 Multivariate regression analysis of neonatal hyperbilirubinemia

Variable	Adjusted OR (95% CI)	P
<i>Sex of baby</i>		
Female	Referent	
Male	1.26 (0.69–2.31)	0.459
GA (weeks)	0.80 (0.59–1.07)	0.134
<i>Cesarean delivery</i>		
No	Referent	
Yes	0.27 (0.10–0.73)	0.010
BW at birth	1.00 (0.998–1.001)	0.288
Maximal body weight loss (%)	1.25 (1.11–1.41)	<0.001
<i>UGT1A1 gene-coding region 211</i>		
G/G	Referent	
G/A	1.76 (0.94–3.28)	0.075
A/A	1.18 (0.13–10.84)	0.881
<i>UGT1A1 gene promoter</i>		
(TA) ₆ /(TA) ₆	Referent	
(TA) ₆ /(TA) ₇	0.37 (0.15–0.89)	0.027
(TA) ₇ /(TA) ₇	—	—

Abbreviations: BW, birth weight; CI, confidence interval; GA, gestational age; OR, odds ratio.

Table 5 Subgroup analysis of *UGT1A1* gene-coding region 211 genotypes and various levels of maximal body weight loss

<i>UGT1A1</i> gene-coding region 211.					
Stratifying variable	Adjusted OR (95% CI) ^a				
	G/G	G/A	P	A/A	P
<i>Maximal body weight loss</i>					
<5.0%	Referent	—	—	—	—
$\geq 5.0\%$	Referent	2.23 (1.21–4.11)	0.010	1.86 (0.2–17.41)	0.588
<i>Maximal body weight loss</i>					
<7.5%	Referent	1.00 (0.30–3.34)	0.990	—	—
$\geq 7.5\%$	Referent	2.75 (1.35–5.62)	0.005	1.73 (0.18–16.54)	0.634
<i>Maximal body weight loss</i>					
<10%	Referent	1.27 (0.60–2.71)	0.534	1.96 (0.20–18.88)	0.560
$\geq 10\%$	Referent	6.20 (2.08–18.50)	0.0011	—	—

^aAdjustment for birth weight.

between the degree of maximal body weight loss and the G71R genotype of the *UGT1A1* gene was studied. Subgroup analysis revealed that the G71R mutation was a significant risk factor for the development of neonatal hyperbilirubinemia in the neonates with 5.0% or greater maximal body weight loss (Table 5). The influence of the G71R mutation increased in parallel with the degree of maximal body weight loss. Neonates who carried heterozygous G71R mutation of the *UGT1A1* gene showed a relative risk (OR: 2.23; 95% CI: 1.21–4.11) for the development of neonatal hyperbilirubinemia in infants with a 5.0% or greater maximal body weight loss; whereas the corresponding OR (95% CI) was 2.75 (1.35–5.62) in infants with a 7.5% or greater maximal body weight loss and the OR (95% CI) was 6.2 (2.08–18.50) in infants with a 10.0% or greater maximal body weight loss. Data on homozygous G71R mutation did not show significant difference possibly because of the small size of samples.

DISCUSSION

We studied the association of G71R and (TA)₇ mutations and hyperbilirubinemia of breast-fed Japanese neonates, and found that maximal body weight loss during the neonatal period is the only independent risk factor for the development of neonatal hyperbilirubinemia, and G71R mutation is a significant risk factor only in neonates with a 5.0% or greater maximal body weight loss.

Neonatal hyperbilirubinemia is a physiological phenomenon, but its severity is also affected by genetic factors. We previously reported that G71R mutation of the *UGT1A1* gene was prevalent in Japanese, Koreans and Chinese and was associated with neonatal hyperbilirubinemia.^{4,5} However, half of the Japanese neonates with severe hyperbilirubinemia did not carry the G71R mutation. To identify other predisposing factors of neonatal hyperbilirubinemia, we studied the relation with the enhancer sequence (–3483/–3194) (phenobarbital response enhancer

module) of the *UGT1A1* gene, polymorphic (GT)_n repeats in the promoter region of the hemeoxygenase-1 (a rate-limiting enzyme in heme metabolism) gene, and the composition of fetal hemoglobin (a probable major source of bilirubin in neonates).^{12,13} However, we could not identify any other significant genetic risk factors except the G71R mutation of *UGT1A1*. Recently, a significant association of G71R mutation with hyperbilirubinemia in breast-fed neonates was reported.^{7,8} We classified the full-term breast-fed neonates into two groups depending on the degree of maximal body weight loss. Cesarean delivery and a short pregnancy period were frequently observed in neonates of group B. Cesarean delivery may cause a delayed onset of lactation, leading to an increase in the maximal body weight loss.¹⁴ However, multivariate regression analysis revealed that cesarean delivery and heterozygous (TA)₆ mutation rather decreased the risk of neonatal hyperbilirubinemia. Chang *et al.*⁸ reported that breast-fed neonates with vaginal delivery have a high risk for the development of hyperbilirubinemia. They speculated that vaginal delivery might be associated with oxytocin usage, vacuum extraction and cephalohematoma. Oxytocin exposure is a risk factor for hyperbilirubinemia, which may have some direct effect on neonatal bilirubin metabolism.^{15,16} The association of heterozygous (TA)₆ mutation with a decrease in the risk for hyperbilirubinemia is unknown.

Our findings indicate that maximal body weight loss, that is, inadequate feeding, is an independent risk factor for the development of neonatal hyperbilirubinemia in breast-fed neonates, and the effect of G71R on neonatal hyperbilirubinemia is significant only in infants under inadequate feeding. Chang *et al.*¹⁷ reported that neonates with greater weight loss percentage were associated with hyperbilirubinemia. An 8% or greater weight loss after 48 h and an 11% or greater weight loss after 72 h significantly increased a risk for hyperbilirubinemia. Ishihara *et al.*¹⁸ demonstrated that healthy individuals with *UGT1A1* mutation presented with a significant increase in serum bilirubin levels compared with individuals without any mutation after caloric restriction. Inadequate feeding of breast-fed neonates may increase intestinal bilirubin absorption, impair hepatic conjugation and/or excretion of bilirubin because of energy deficiency, and cause hyperbilirubinemia in neonates carrying the G71R mutation. The American Academy of Pediatrics recommends that clinicians should advise mothers to nurse their infants at least 8–12 times per day for the first several days.¹⁹ Our data suggest that adequate feeding could overcome the genetic predisposing factor (G71R mutation of *UGT1A1*) of neonatal hyperbilirubinemia even in breast-fed infants.

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