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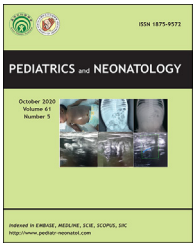

Original Article

Effects of variation status and enzyme activity for UDP-glucuronosyltransferase *1A1* gene on neonatal hyperbilirubinemia

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variation status

Background: We found that Taiwanese adults carrying genotypes of UDP-glucuronosyltransferase (UGT) *1A1* with enzyme activity ≤40% of normal were at high risk for developing Gilbert's syndrome. However, the relationship between UGT1A1 activity and neonatal hyperbilirubinemia has never been evaluated for Taiwanese.
Methods: We enrolled 141 hyperbilirubinemic neonates partially fed supplementary infant formula and 432 controls; and 112 hyperbilirubinemic neonates exclusively breastfed and 493 controls. The five single nucleotide polymorphisms (SNPs) at nucleotides −53, 211, 686, 1091 and 1456 in the *UGT1A1* gene were determined and UGT1A1 activity was estimated. Odds ratios (ORs) of variation status in the *UGT1A1* gene and enzyme activity for the development of neonatal hyperbilirubinemia were calculated, respectively.
Results: For neonates partially fed supplementary infant formula, the adjusted OR (AOR) for the development of hyperbilirubinemia was significantly higher in the neonates carrying the homozygous variation (211AA) in the *UGT1A1* gene than for those carrying the wild type (AOR = 6.00, $p < 0.001$). Only the AOR of those carrying UGT1A1 activity ranked 31–40% of normal was statistically significant (AOR = 3.16, $p < 0.001$). For the hyperbilirubinemic neonates exclusively breastfed, AOR for the development of hyperbilirubinemia is positively correlated to degree of variation (AOR = 1.95, 2.19 and 4.53; with $p = 0.003$, 0.05 and < 0.001, respectively), while the effect of UGT1A1 enzyme activity was varied (AOR = 1.02–3.72, with $p = 0.95 \sim < 0.001$).
Conclusion: The estimated enzyme activity depending on combination of SNPs (genotypes) in the *UGT1A1* gene could not be utilized to explain the development of neonatal hyperbilirubinemia. We

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reconfirm that the −53 A(TA)$_7$TAA/A(TA)$_7$TAA is not, while the 211AA is a risk factor for the development of neonatal hyperbilirubinemia in Taiwanese.

1. Introduction

The UDP-glucuronosyltransferase 1A1 (UGT1A1), a 533-amino acid protein synthesized in liver is the sole enzyme responsible for the glucuronidation of bilirubin in humans.[1] It is encoded by the *UGT1A1* gene which is located on chromosome 2q37.1.[1] A number of studies have reported that certain single nucleotide polymorphisms (SNPs) in the *UGT1A1* gene differ among ethnic groups.[2,3] For example, the A(TA)$_7$TAA/A(TA)$_7$TAA variation (UGT1A1*28, rs8175347) at nucleotide −53 is the main cause of nonhemolytic unconjugated hyperbilirubinemia in neonates and Gilbert's syndrome in Caucasians,[2,4–6] while in Asians homozygous variation at nucleotide 211 (G > A, 211AA) (p.Gly71Arg, UGT1A1*6, rs4148323) is the main cause of neonatal hyperbilirubinemia and both 211AA and A(TA)$_7$TAA/A(TA)$_7$TAA are main causes of Gilbert's syndrome.[3,6–8]

For general populations and subjects suffering from Gilbert's syndrome in Asians, five SNPs of *UGT1A1* gene have been reported[9–11]: c.−53 A(TA)$_6$TAA > A(TA)$_7$TAA, c.211 G > A, c.686 C > A (p.Pro229Gln, UGT1A1*27, rs35350960), c.1091 C > T (p.Pro364Leu, UGT1A1*63, rs34946978) and c.1456 T > G (p.Tyr486Asp, UGT1A1*7, rs34993780). Thus a large number of *UGT1A1* genotypes may occur. Grouping on the basis of UGT1A1 activity rather than on the basis of genotypes may reduce the number of potential subgroups, thereby simplifying the analysis of relationship between *UGT1A1* gene and certain diseases.[11] Very recently, we found that Taiwanese carrying genotypes with UGT1A1 activity ≤40% of normal are at high risk for developing Gilbert's syndrome.[11] In contrast, the relationship between UGT1A1 activity and neonatal hyperbilirubinemia has never been evaluated for Taiwanese.

Immaturity of the conjugation system for bilirubin (UGT1A1) is universal in neonates,[12] while SNPs in the *UGT1A1* gene differ among ethnic groups.[2,3] Therefore, it seems necessary to comprehensively analyze genetic risk-factors for *UGT1A1* gene for the development of neonatal hyperbilirubinemia in every ethnic group. In this study, we examined the five SNPs of *UGT1A1* gene to investigate the effects of variation status and enzyme activity in *UGT1A1* gene on Taiwanese neonates suffering from hyperbilirubinemia.

2. Materials and methods

2.1. Study subjects

The subjects of this case−control study were obtained from Far Eastern Memorial Hospital, New Taipei City, Taiwan. Written informed consent was obtained from all participants' parents and the study protocol was approved by the research committee of Far Eastern Memorial Hospital (IRB numbers 100152-F and 101026-F), following the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

Umbilical cord blood samples were collected for neonatal screening of *UGT1A1* gene. Gender of every study subject was recorded. In our study, the neonates were divided into two groups; one was supplemented by infant formula partially and the other was exclusively breastfed. Those babies with risk factors for neonatal hyperbilirubinemia, such as ABO incompatibility, glucose-6-phosphate dehydrogenase (G6PD) deficiency, hemolytic anemia, hypoxia/asphyxia, dehydration/vomiting, cephalohematoma, sepsis, liver dysfunction, hypothyroidism, and small for gestational age babies were excluded. In the meantime, the analyses of total serum bilirubin levels were obtained on the third day (between 64 and 72 h postnatal life) before they were discharged from the hospital. A return visit was recommended for all neonates. The follow-up total serum bilirubin levels were obtained within 24−48 h after discharge or later if indicated. All neonates received outpatient follow-up until bilirubin levels declined. Neonatal hyperbilirubinemia was diagnosed if a neonate had a serum bilirubin level ≥256.5 μmol/L (15.0 mg/dL) within 1 week after birth.

2.2. Determinations of SNPs and UGT1A1 enzyme activity

Genomic DNA was isolated from whole blood cells using a blood DNA isolation kit (Maxim Biotech Inc., San Francisco, CA, USA). The five SNPs at nucleotides −53, 211, 686, 1091 and 1456 of the *UGT1A1* gene were determined by means of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), as previously described.[11] The DNAs of the five known variants in the *UGT1A1* gene, which had been found in Taiwanese and identified by DNA sequencing methods,[11] were run as positive controls in each performance of genotyping assays.

Study subjects were divided into categories of wild type, heterozygous variation, compound heterozygous variations and homozygous variation depending on variation status of *UGT1A1* gene and classified as one of the 10 subgroups (Q1 ~ Q10, by using 10% of normal as an interval) according to their UGT1A1 enzyme activity, respectively. The enzyme activity values for 686CA, −53 A(TA)$_7$TAA/A(TA)$_7$TAA, 1091 TT, 211 GA, 211AA and 1456 GG variants were obtained from previously published studies[4,8,13,14] as shown in Table 1, while those for 686AA, −53 A(TA)$_6$TAA/A(TA)$_7$TAA, 1091CT and 1456 TG variants were estimated by calculation

{UGT1A1 activity for 686AA = [(activity for 686CA)^2 (square)], while activity for A(TA)$_6$TAA/A(TA)$_7$TAA = [activity for A(TA)$_7$TAA/A(TA)$_7$TAA]$^{1/2}$ and so on}.[11] For subjects carrying multiple SNPs in the *UGT1A1* gene, estimated UGT1A1 enzyme-activities were obtained by calculations.[11] However, for subjects carrying two SNPs in close linkage, e.g., 686A/−53 A(TA)$_7$TAA, the UGT1A1 enzyme activity of the SNP with lower activity between those two SNPs was estimated, avoiding over- or under-estimation.[11]

2.3. Statistical analysis

The odds ratio (OR) for the development of neonatal hyperbilirubinemia was assigned 1.0 for (number of patients carrying the wild type of *UGT1A1* gene)/(number of controls carrying the wild type of *UGT1A1* gene) or for (number of patients in Q10)/(number of controls in Q10), respectively. The Mantel-Haenszel chi-square test was then used to calculate the OR and the 95% confidence interval (CI) for subjects with different variations or for subjects with genotypes in subgroups Q1∼Q9, respectively. A 95% CI of the OR above or below 1.0 or a p value < 0.05 was defined as constituting statistical significance. All statistical analyses were performed with the statistical package SPSS for Windows (Version 18.0, SPSS Inc., Chicago, IL).

3. Results

3.1. Variation status and UGT1A1 activities

The positive controls of the wild-type, heterozygote and homozygote for each of the five SNPs determined were accurate in each assay of PCR-RFLP. Numbers of the case group and the control group we enrolled were 141 and 432 for the neonates who were supplemented by infant formula partially; and 112 and 493 for the neonates who were exclusively breastfed.

Variation status and UGT1A1 activities for the case and control groups are presented as in Tables 2 and 3. For the neonates who were supplemented by infant formula partially, gender distribution was significantly different between the case group and the control group [(male neonates)/(female neonates) ratio = 91/50 versus 344/88, $p < 0.001$ by two sample *t* test]. Therefore, it was necessary to adjust the OR for the development of neonatal hyperbilirubinemia. Among the 141 neonates with hyperbilirubinemia, 2.1% (3/141) and 11.3% (16/141) carried A(TA)$_7$TAA/A(TA)$_7$TAA and 211AA, respectively. For the neonates who were exclusively breastfed, gender distribution was significantly different between the case and the control groups [(male neonates)/(female neonates) ratio = 88/24 versus 261/232, $p < 0.001$ by two sample *t* test]. Among the 112 neonates with hyperbilirubinemia, none and 8.0% (9/112) carried A(TA)$_7$TAA/A(TA)$_7$TAA and 211AA, respectively.

Table 1 Variation status of the five polymorphisms and their UGT1A1 activities.

Nucleotide		UGT1A1 activity, % of normal	Reference
686	CA	37	13
	AA	$(37\%)^2 = 14$[a]	
−53	(TA)$_6$TAA/A(TA)$_7$TAA	$(25.5\%)^{1/2} = 50$[a]	
	A(TA)$_7$TAA/A(TA)$_7$TAA	25.5	4
1091	CT	$(36\%)^{1/2} = 60$[a]	
	TT	36	8
211	GA	60	14
	AA	32.2	14
1456	TG	$(7.6\%)^{1/2} = 27.6$[a]	
	GG	7.6	14

UGT, UDP-glucuronosyltransferase.
[a] UGT1A1 activity: estimated value.

3.2. Adjusted OR for the neonates supplemented by infant formula partially

As shown in Table 2, the adjusted OR (AOR) for the neonates carrying homozygous variation in the *UGT1A1* gene was significantly higher than for those carrying the wild type (AOR = 6.00, $p < 0.001$), while the AORs for those carrying heterozygous variation and compound heterozygous variations were non-significant. When the neonates were classified as one of the 10 subgroups (Q1∼Q10) according to the UGT1A1 enzyme activity, no subject carried Q9, Q8, Q7 and Q1. Table 2 shows that neonates of the Q4 subgroup had a significant AOR (3.16, $p < 0.001$) for the development of neonatal hyperbilirubinemia, while those carrying Q6, Q5 and Q3 had a non-significant AOR and only one neonate of the control group carried Q2 genotype.

3.3. Adjusted OR for the exclusively-breastfed neonates

After adjustment, the AOR for the neonates carrying heterozygous variation, compound heterozygous variations and homozygous variation in the *UGT1A1* gene were significantly higher than for those carrying the wild type (AOR = 1.95, 2.19 and 4.53 with p = 0.003, 0.05 and < 0.001, respectively) (Table 3). As shown in Table 3, the subjects carrying Q6, Q4 and Q3 had a significant AOR (2.39, 2.50 and 3.72 with p = $<$0.001, 0.013 and 0.029, respectively) for the development of neonatal hyperbilirubinemia, while those carrying Q5 and Q2 had a non-significant AOR.

4. Discussion

For neonatal hyperbilirubinemia, although homozygous variation in the *UGT1A1* gene is observed as a risk factor in this study, most (16/19 for the neonates who were supplemented by infant formula partially; and all for the nine neonates who were exclusively breastfed) of the neonates carrying the homozygous variation possess 211AA, not A(TA)$_7$TAA/A(TA)$_7$TAA. Such a finding supports that *UGT1A1* 211G > A polymorphism significantly increases the risk of neonatal hyperbilirubinemia in the Asian population, while the A(TA)$_7$TAA/A(TA)$_7$TAA is the main genetic

Table 2 Variation status, UGT1A1 activity and odds ratio for the development of hyperbilirubinemia in the neonates who were supplemented by infant formula partially.

	UGT1A1 activity % of normal	Case n = 141[a]	Control n = 432[b]	OR (95% CI) [P[c]]	AOR (95% CI) [P[c]]
Wild type	100	52	204	1.0	1.0
Heterozygous variation		60	187	1.26 (0.83−1.92) [0.284]	1.28 (0.84−1.97) [0.252]
6/7	50	14	71		
211 GA	60	45	115		
1091CT	60	1	1		
Compound heterozygous variation		10	28	1.40 (0.64−3.07) [0.397]	1.39 (0.63−3.08) [0.414]
6/7, 211 GA	30	5	7		
6/7, 686CA	37	4	13		
6/7, 1091CT	30	0	5		
6/7, 211 GA, 686CA	22.2	1	2		
6/7, 211 GA, 1091CT	18	0	1		
Homozygous variation		19	13	5.73 (2.66−12.36) [<0.001]	6.00 (2.75−13.09) [<0.001]
7/7	25.5	3	1		
211AA	32.2	16	12		
Q10	100	52	204	1.0	1.0
Q6	51−60	46	116	1.56 (0.99−2.46) [0.057]	1.58 (0.99−2.52) [0.053]
Q5	41−50	14	71	0.77 (0.40−1.48) [0.437]	0.78 (0.40−1.50) [0.450]
Q4	31−40	20	25	3.14 (1.62−6.09) [<0.001]	3.16 (1.64−6.11) [<0.001]
Q3	21−30	9	15	2.35 (0.98−5.68) [0.051]	2.36 (0.98−5.70) [0.052]
Q2	11−20	0	1	−	−

AOR, adjusted odds ratio; CI, confidence interval; OR, odds ratio; UGT, UDP-glucuronosyltransferase; 6/7, $A(TA)_6TAA/A(TA)_7TAA$; 7/7,$A(TA)_7TAA/A(TA)_7TAA$.

[a] 91 male and 50 female neonates.

[b] 344 male and 88 female neonates.

[c] Calculated by Mantel−Haenszel chi-square test.

factor for causing neonatal hyperbilirubinemia in Caucasians.[6,7,15−21] These data show that the variation of $A(TA)_6TAA > A(TA)_7TAA$ at nucleotide −53 in the *UGT1A1* gene plays different role for the development of neonatal hyperbilirubinemia in Asians and Caucasians.

Moreover, there is an inverse association between the TA7 repeat variant and hyperbilirubinemia risk in Chinese neonates[16,22] and in Japanese neonates,[23] respectively. For example, allele frequency of the $A(TA)_6TAA > A(TA)_7TAA$ in Chinese hyperbilirubinemic neonates was 0.054, which was different from that (0.157) in the controls.[16] In one of our previous studies, we found that allele frequency of the $A(TA)_6TAA > A(TA)_7TAA$ was significantly different between neonates with severe hyperbilirubinemia [peak serum bilirubin value within 1 week of life ≥342 μmol/L (20.0 mg/dL)] and the healthy controls (0.042 versus 0.120).[15] One study in Taiwanese neonates also reported a dose-dependent effect of the $A(TA)_6TAA > A(TA)_7TAA$ on the lower serum bilirubin levels (bilirubin values: homozygote < heterozygote < wild type).[19] Putting these data together, the TA7 repeat variant of *UGT1A1* gene seems to have a protective effect on hyperbilirubinemia development in Asian neonates fed with breast milk. It was hypothesized that the (TA)n repeat might be a balanced polymorphism evolutionarily selected to maintain serum bilirubin in an optimal range in the face of largely undefined genetic and environmental pressures,[24] then the promoter variant may show different, even opposite, effects on serum bilirubin level.[16] Breast milk suppresses UGT1A1 expression in the small intestine[24] and such an undefined environmental pressure induces the effect of (TA)n repeat on maintaining serum bilirubin. We think this mechanism is probably, at least partially, the reason the TA7 repeat variant having a protective effect on hyperbilirubinemia development in Asian neonates fed with breast milk.

In our previous study, we found that both variation status and enzyme activity of *UGT1A1* gene were associated with the causing of Gilbert's syndrome in adult Taiwanese.[11] Moreover, that study showed that risk for the development of Gilbert's syndrome was positively correlated with degree of variation and inversely correlated with UGT1A1 activity and subjects carrying the genotypes with UGT1A1 activity ≤20% of normal suffer from Gilbert's syndrome.[11] However, in this study, when enzyme activity of UGT1A1 is concerned, it seems

Table 3 Variation status, UGT1A1 activity and odds ratio for the development of hyperbilirubinemia in the neonates who were exclusively breastfed.

	UGT1A1 activity % of normal	Case n = 112[a]	Control n = 493[b]	OR (95% CI) [P[c]]	AOR (95% CI) [P[c]]
Wild type	100	44	286	1.0	1.0
Heterozygous variation		50	167	1.95 (1.24–3.05) [0.003]	1.95 (1.24–3.06) [0.003]
6/7	50	8	51		
211 GA	60	40	107		
1091 CT	60	2	7		
1456 TG	27.6	0	2		
Compound heterozygous variation		9	27	2.17 (0.96–4.91) [0.059]	2.19 (1.00–4.93) [0.05]
6/7, 211 GA	30	3	1		
6/7, 686CA	37	1	16		
6/7, 1091CT	30	1	0		
6/7, 211 GA, 686CA	22.2	0	4		
211 GA, 1091CT	36	2	2		
211 GA, 1456 TG	17	1	0		
1091CT, 1456 TG	17	1	4		
Homozygous variation		9	13	4.50 (1.82–11.15) [<0.001]	4.53 (1.84–11.18) [<0.001]
211AA	32.2	9	13		
Q10	100	44	286	1.0	1.0
Q6	51–60	42	114	2.39 (1.49–3.85) [< 0.001]	2.39 (1.50–3.82) [< 0.001]
Q5	41–50	8	51	1.02 (0.45–2.29) [0.96]	1.02 (0.45–2.30) [0.95]
Q4	31–40	12	31	2.52 (1.20–5.26) [0.012]	2.50 (1.18–5.23) [0.013]
Q3	21–30	4	7	3.71 (1.04–13.21) [0.031]	3.72 (1.05–13.23) [0.029]
Q2	11–20	2	4	3.25 (0.58–18.27) [0.416 (Yates correction)]	3.23 (0.56–18.25) [0.418]

AOR, adjusted odds ratio; CI, confidence interval; OR, odds ratio; UGT, UDP-glucuronosyltransferase; 6/7, $A(TA)_6TAA/A(TA)_7TAA$; 7/7,$A(TA)_7TAA/A(TA)_7TAA$.

[a] 88 male and 24 female neonates.

[b] 261 male and 232 female neonates.

[c] Calculated by Mantel–Haenszel chi-square test.

impossible to explain the mechanism of hyperbilirubinemia in neonates based on UGT1A1 activity only depending on *UGT1A1* genotypes. It has been verified that delayed expression of UGT1A1 during the early stages of neonatal development is a tightly controlled event involving coordinated intrahepatic and extrahepatic (e.g., small intestine) regulation.[17,24] The results of studies, by using humanized *UGT1* mice fed with breast milk as the animal model, reveal that regulation of intestinal UGT1A1 during neonatal development appeared to play an important role in bilirubin glucuronidation and elimination when liver gene expression is compromised.[17,24] Incomplete expression of UGT1A1 in neonates is probably the reason, at least in part, why the relationship between UGT1A1 activity and the development of neonatal hyperbilirubinemia is not clear.

Although UGT1A1 is the sole enzyme responsible for the glucuronidation of bilirubin in humans, low UGT1A1 expression (activity) is not the sole risk factor for the development of unconjugated hyperbilirubinemia.[2,16,17,19] Indeed, in addition to defective bilirubin conjugation (low UGT1A1 activity), such as defective bilirubin uptake by hepatocytes (e.g., polymorphisms in the organic anion transporting polypeptide 2), defective heme oxygenases, defective biliverdin reductase A, G6PD deficiency and breast feeding are also risk factors for the development of unconjugated hyperbilirubinemia.[2,16,17,19] Those risk factors are worthy of investigation in neonates suffering from hyperbilirubinemia and the cause is not attributable to variations in the *UGT1A1* gene.

There are some limitations in this study. First, only Taiwanese were enrolled. Nevertheless, the main findings of our results are in agreement with random studies reported by Japanese and Chinese.[16–18,20,22,23] Therefore, this study may provide important references for Asian populations. Second, the further factor for the development of hyperbilirubinemia, the variation at nucleotide

−3279 (T > G) (UGT1A1∗60, rs4124874) in the *UGT1A1* gene,[11,25] was ignored. This SNP will be examined in our further study on neonatal hyperbilirubinemia. Third, in our study, the neonates were divided into two groups; one was supplemented by infant formula partially and the other was exclusively breastfed. However, the amount of total feeding and supplement formula were not recorded. This may be a confounding factor to affect the results. The trend shows that the enzyme activity below 50% (Q5) has an elevated AOR in supplement by formula and exclusively breastfed infants except in the Q6 and Q2 subgroup in breastfed infants. In Taiwanese infants, Q2 is rare and Q6 may be influenced by other confounding factors (breast milk amount). Fourth, we did not follow the seven infants in Q2 subgroup. Therefore, we do not know if the enzyme activity improved or not after infant growth. It is an interesting issue because the hyperbilirubinemia almost recovered after neonatal stage. Fifth, there are no demographic data about the gestational age, birth weight and birth mode. As we know, those factors influence the bilirubin level. Sixth, some infants may be missing during the first week's follow-up. Nevertheless, those confounding factors should not influence the main findings of this study.

In conclusion, for Taiwanese, the estimated enzyme activity depending on genotypes of *UGT1A1* could not be utilized to explain the development of neonatal hyperbilirubinemia. Neonatal hyperbilirubinemia is a multifactorial process and is not so closely related to enzyme activity. However, we confirmed that the 211AA corresponding to 31−40% of UGT1A1 activity is a risk factor for the development of neonatal hyperbilirubinemia in Taiwanese, while $A(TA)_6TAA > A(TA)_7TAA$ variant may be a protective factor for suffering from hyperbilirubinemia in neonates fed with breast milk. Our results reveal that neonatal hyperbilirubinemia in Taiwanese is related to 211 G > A variant in the *UGT1A1* gene, while such a variant is very rare in Caucasians.[6] Therefore, we recommend that information about neonatal hyperbilirubinemia and breastfeeding hyperbilirubinemia could be incorporated into prenatal education for mothers who intend to breastfeed, as other Taiwanese authors reported recently.[26] Furthermore, we agree that further study may be needed to target women who stopped breastfeeding because of breastfeeding hyperbilirubinemia specifically.[26]

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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