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Impact of *G-6-PD*, *SLCO1B1* and *UGT1A1* variants on severity of neonatal hyperbilirubinemia in Northeastern Thailand

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KEYWORDS

G-6-PD; *SLCO1B1*; *UGT1A1*; Northeastern Thailand; Neonatal hyperbilirubinemia.

ABSTRACT

Neonatal hyperbilirubinemia is a common complication in Thailand. The polymorphisms of *SLCO1B1* (encoding solute carrier organic anion transporter 1B1) and *UGT1A1* (uridine diphosphate glucuronosyltransferase 1A1) as well as *G-6-PD* mutations associated with glucose-6-phosphate dehydrogenase deficiency have been reported as genetic risk factors for this condition. This study investigated the association between these genetic variations with severity of neonatal hyperbilirubinemia in northeastern Thai newborns. Neonates (n = 204) with hyperbilirubinemia were analyzed for common *G-6-PD* mutations and polymorphisms of *SLCO1B1* c.388G>A, *SLCO1B1* c.521T>C and *UGT1A1* g.-3279T>G using restriction fragment length polymorphism-PCR assay. *G-6-PD* mutations are significant genetic risk factors for severe neonatal hyperbilirubinemia indicated by significantly higher peak total serum bilirubin (coefficient = 0.93, 95% CI: 0.22-1.64, *p-value* = 0.011), longer duration of phototherapy (coefficient = 14.45, 95% CI: 6.92-21.99, *p-value* = 0.0001), early (≤48 hours) onset of hyperbilirubinemia (OR = 2.29, 95% CI: 1.22-4.31, *p-value* = 0.010) and more hospital readmission (OR = 4.13, 95% CI: 1.09-15.67, *p*-value = 0.037). *SLCO1B1* c.388G>A, *SLCO1B1* c.521T>C and *UGT1A1* g.-3279T>G polymorphisms were present in northeastern Thai neonates with allele frequencies similar to those of other Asian populations, but they were not associated with severity of neonatal hyperbilirubinemia. These findings indicate that if genetic factors impacting on neonatal hyperbilirubinemia are to be more fully understood, a larger cohort study of these genetic variations and other pertinent genes involved in neonatal bilirubin will be needed.

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Introduction

Hyperbilirubinemia (jaundice) is a common clinical manifestation of newborns during the first week of life, associated with a variety of maternal and neonatal factors, such as blood group (ABO or Rh) incompatibility, prematurity, birth trauma, infection, Asian ethnicity, breastfeeding, maternal diabetes, and previous sibling with hyperbilirubinemia[1]. However, in the majority of cases, the underlying cause of hyperbilirubinemia remains unidentified, but gene variants have been increasingly recognized as risk factors of neonatal hyperbilirubinemia.

Bilirubin is produced mainly from degradation of hemoglobin in senescent erythrocytes. The free (unconjugated) form of bilirubin is water-insoluble and is bound to albumin in the plasma. It is taken up by hepatocytes through predominantly organic anion transporter polypeptide 1B1 (OATP1B1) [encoded by *solute carrier organic anion transporter family member 1B1* (*SLCO1B1*)] and then conjugated with glucuronic acid by uridine 5'diphospho (UDP)glucuronosyltransferases (UGT) [encoded by *UDP-glucuronosyltransferase 1A1* (*UGT1A1*)], before being secreted into bile[2]. Therefore, variations of genes involved in bilirubin metabolism as well as erythrocyte life span, such as *G-6-PD*, are important candidate genes associated with neonatal hyperbilirubinemia. Moreover, two genome-wide association studies (GWAS) for serum bilirubin metabolism identified three loci of significance, namely, *UGT1A1*, *SLCO1B1* and *G-6-PD*[3, 4].

G-6-PD is the rate-limiting enzyme of the pentose phosphate pathway, which provides reduced nicotinamide adenine dinucleotide phosphate (NADPH) essential for all cells including erythrocytes. Limited production of NADPH increases susceptibility of erythrocytes to oxidative stress, which may shorten their life span and lead to low-grade hemolysis causing an increase in bilirubin production[5]. G-6-PD deficiency is considered as risk factor of neonatal hyperbilirubinemia in the American Academy of Pediatrics clinical practice guideline for management of hyperbilirubinemia in newborns[6]. G-6-PD deficiency is highly prevalent in malaria-endemic regions including Africa and Southeast Asia[7]. In Thailand, prevalence of G-6-PD deficiency among neonates with hyperbilirubinemia is as high as 22.1% in males and 10.1% in females[8].

Of the genes involved in bilirubin metabolism, *UGT1A1* has been widely studied as it is the key enzyme for bilirubin conjugation. Three major polymorphisms, namely insertion of TA (TA_7) in promoter TATA box, c.211G>A and g.-3279T>G, have been identified in different populations. Meta-analysis showed TA_7 and c.211G>A mutations significantly increase the risk of neonatal hyperbilirubinemia in both Caucasian and Asian populations[9]. However, more recent meta-analyses have reported different conclusions: Mehrad-Majd *et al*. confirmed c.211G>A polymorphism significantly increases the risk of neonatal hyperbilirubinemia in Asian population, but results in Caucasian population require further well-designed epidemiological investigation[10]. Li and Zhang demonstrated TA_7 polymorphism may not be associated with risk of neonatal hyperbilirubinemia[11]. In 2002, Sugatani *el al*. identified another polymorphic mutation, g.-3279T>G, in the *UGT1A1* promotor, located in phenobarbital responsive enhancer module (gtPBREM) and reduces transcriptional activity of *UGT1A1* promotor by 60%[12]. Several other studies demonstrated g.-3279T>G polymorphism is a genetic risk factor for neonatal hyperbilirubinemia[13-15].

Variations in *SLCO1B1* may predispose individuals to hyperbilirubinemia due to limitation in hepatic bilirubin uptake[16]. A recent meta-analysis demonstrated c.388G>A polymorphism is a risk factor for developing neonatal hyperbilirubinemia in Chinese neonates, but not in white, Thai, Brazilian, or Malaysians populations, and c.521T>C variant provides protection for neonatal hyperbilirubinemia in Chinese neonates, but not in white, Thai, Brazilian, or Malaysian populations[17].

Neonatal hyperbilirubinemia occurs more frequently and with greater severity among Asians, especially Southeast and Far East Asians, including Thais[18]. In Thailand, studies on genetic risk factors of neonatal hyperbilirubinemia are limited. Prachukthum *et al*. reported G-6-PD deficiency and *UGT1A1* c.211G>A, but not *SLCO1B1*

c.388G>A, polymorphisms are associated with neonatal hyperbilirubinemia[19]. In addition, in northeast Thailand *UGT1A1* c.211G>A and TA_7 promotor mutations are associated with higher peak total serum bilirubin (TSB) in G-6-PD deficient neonates with hyperbilirubinemia[20]. However, prevalence of *SLCO1B1* c.388G>A, *SLCO1B1* c.521T>C and *UGT1A1* g.-3279T>G polymorphisms have not been investigated in northeastern Thailand. Here, prevalence of these polymorphisms and impact of these gene variants as well as *G-6-PD* variants on severity of neonatal hyperbilirubinemia in northeastern Thailand were investigated.

Materials and methods

Study subjects

Based on previous studies on prevalence of genetic polymorphisms in Southeast Asia,[13, 19, 21] sample size was statistically calculated at 204. Leftover EDTA blood samples of near full term and full term neonates (gestational age 35 - 42 weeks) with hyperbilirubinemia were collected from the Diagnostic Microscopy Unit, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand from October 2016 to March 2018. Neonatal hyperbilirubinemia is defined according to the American Academy of Pediatrics Guidelines 2004[6]. Demographic and clinical data of subjects acquired from medical records by a pediatrician were gender, gestation age, birth weight, delivery method, type of feeding (exclusive breast-feeding, formula or combined), percent weight loss, and peak TSB. Neonates with known risk factors of hyperbilirubinemia, viz. gestational age <35 weeks, birth weight <2000 g, cephalhematoma, ABO incompatibility, maternal diabetes, and infection were excluded. The study protocol was approved by the Institutional Review Board of Khon Kaen University (HE591531).

Molecular analysis of gene variants

Genomic DNA was isolated from whole blood using DNAzol reagent kit (Invitrogen, Carlsbad, USA); concentration and purity ($A_{260\ nm}/A_{280\ nm}$ = 1.8 - 2.0) were determined employing an Eppendorf BioSpectrometer (Eppendorf, Hamburg, Germany) and adjusted to 10 ng/µL. PCR-restriction fragment length polymorphism (RFLP) assay was carried out to detect common *G-6-PD* mutations and *SLCO1B1* c.388G>A, *SLCO1B1* c.521T>C and *UGT1A1* g.-3279T>G polymorphisms. The seven common *G-6-PD* variants present in northeastern Thai population, namely, *G-6-PD* Canton (c.1376G>T, Arg459Leu), Chinese-4 (c.392G>T, Gly131Val), Chinese-5 (c.1024C>T, Leu342Phe), Kaiping (c.1388G>A, Arg463His), Mahidol (c.487G>A, Gly163Ser), Union (c.1360C>T, Asp454Cys), and Viangchan (c.871G>A, Val291Met), were detected as previously described[8, 22]. Identification of *SLCO1B1* c.388G>A, *SLCO1B1* c.521T>C and *UGT1A1* g.-3279T>G polymorphisms was performed in a 50-µL reaction mixture containing 10x ASPCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 30 mM $MgCl_2$, and 0.01% gelatin), 1.0 U *Taq* DNA polymerase (New England Biolabs, Ipswich, USA), 20 ng of each primer,[19, 23, 24] 10 mM dNTPs and 50 ng of DNA template. Thermocycling (conducted in TProfessional thermocycler; Biometra, Gottingen, Germany) conditions were as follows: 94°C for 5 minutes; 35 cycles of 94°C for 1 minute, 60°C (for *UGT1A1* g.-3279T>G) or 62°C (for *SLCO1B1* c.388G>A and *SLCO1B1* c.521T>C) for 1 minute and 72°C for 1 minute; and a final step of 72°C for 10 minutes. Then, a 5-µL aliquot of PCR solution was incubated with 5 U restriction enzymes (New England Biolabs, Ipswich, USA), *Dra* I for *UGT1A1* g.-3279T>G, *Taq*α I for *SLCO1B1* c.388G>A and *Hha* I for *SLCO1B1* c.521T>C, in a 20-µL reaction solution at 37°C for *Dra* I and *Hha* I or 65°C for *Taq*α I for 2 hours. A 7-µL aliquot from each reaction solution was subjected to 3% agarose gel-electrophoresis, stained with ethidium bromide and visualized under UV illumination. Two samples of wild type, homozygote and heterozygote of each gene polymorphism were randomly selected to be confirmed by DNA sequencing (First BASE Laboratories, Selangor, Malaysia).

Statistical analysis

Statistical analysis was performed using STATA software version 10.1 (StataCorp LLC, College Station, USA). Genotype frequency of each genetic variant was calculated as percent total samples and allele frequency as percent total

alleles. Association between genetic variations and continuous variables including peak TSB and duration of phototherapy were evaluated by multiple linear regression analysis. Multiple logistic regression analysis was applied to test association between genetic variations and categorical variables (onset of hyperbilirubinemia ≤48 hours, requirement of phototherapy and hospital readmission). Significance of association was demonstrated either by coefficient value or odds ratio (OR) with 95% confidence interval (CI). Statistical significance is accepted at *p*-value <0.05.

Results

Demographic and clinical characteristics

Demographic and clinical characteristics of the 204 neonates are presented in Table 1. There were twice as many male (64.7%) as female (35.3%) neonates, with 45.6% delivered by Cesarean-section, and with average gestational age and birth weight within normal limits[6]. However, a minority of neonates (14.2%) suffered >10% weight loss. Almost all infants (98.5%) were exclusively breastfed. Average ± SD peak TSB was 14.8 ± 2.6 mg/dL, indicating hyperbilirubinemia[16] and the majority of neonates (83.8%) required phototherapy.

Frequency of gene variants

Genotype and allele frequencies of gene variations are presented in Table 2. *UGT1A1* g.-3279T>G polymorphism was the most common (G allele frequency 40.4%) in the studied population, followed by *SLCO1B1* c.388G>A polymorphism (A allele frequency 22.3%), then *SLCO1B1* c.521T>C polymorphism (C allele frequency 10.3%). The observed allele frequencies of *UGT1A1* g.-3279T>G, *SLCO1B* c.388G>A and *SLCO1B1* c.521T>C polymorphisms were in Hardy-Weinberg equilibrium (*p*-value 0.51, 0.59 and 0.91, respectively). *G-6-PD* variants were detected in 50.5% of neonates, with 37.2% hemizygotes, 11.8% heterozygotes and 1.5% homozygotes.

Gene variants and severity of neonatal hyperbilirubinemia

Multiple linear regression analysis indicated *G-6-PD* variant is a significant genetic predictor of higher peak TSB (coefficient = 0.93, 95% CI: 0.22-1.64, *p-value* = 0.011) and longer duration of phototherapy (coefficient = 14.45, 95% CI: 6.92-21.99, *p-value* = 0.0001) while *UGT1A1* g.-3279T>G, and *SLCO1B1* c.388G>A and c.521T>C polymorphisms are not significant genetic predictors for these parameters (Table 3).

Multiple logistic regression analysis also confirmed *G-6-PD* variant is a significant genetic risk factor for early onset (≤48 hours) of hyperbilirubinemia (OR = 2.29, 95% CI: 1.22-4.31, *p-value* = 0.010) or hospital readmission due to hyperbilirubinemia (OR = 4.13, 95% CI: 1.09-15.67, *p-value* = 0.037) but not of requirement for phototherapy. *UGT1A1* g.-3279T>G, *SLCO1B1* c.388G>A and c.521T>C polymorphisms are not significant genetic risk factors for any of these conditions (Table 4).

Table 1 Demographic and clinical characteristics of neonates with hyperbilirubinemia in northeastern Thailand (n = 204)

Characteristics	Result
Peak TSB (mg/dl)	14.8 ± 2.6
Gestational age (week)	38.0 ± 1.4
Birth weight (g)	3,083.6 ± 431.7
Sex	
- Male	132 (64.7)
- Female	72 (35.3)
Delivery mode	
- Normal	102 (50)
- Cesarean section	93 (45.6)
- Vacuum extraction	6 (2.9)
- Forceps extraction	1 (0.5)
- No record	2 (1)
Type of feeding	
- Exclusive breast feeding	201 (98.5)
- Combined breast and formula feeding	1 (0.5)
- No record	2 (1)
Body weight loss	
- ≤10%	168 (82.4)
- >10%	29 (14.2)
- No record	7 (3.4)
Phototherapy	
- Yes	171 (83.8)
- No	33 (16.2)

Note: Data are presented as mean ± standard deviation or n (%).

Discussion

The study confirms and expands our previous report on the relationship between G-6-PD deficiency, *UGT1A1*(TA_6/TA_7) and *UGT1A1* c.211G>A with neonatal hyperbilirubinemia in northeastern Thai newborns. We have determined the frequency of *SLCO1B1* c.388G>A and c.521T>C, and *UGT1A1* g.-3279T>G polymorphisms in the northeasternThai newborns with hyperbilirubinemia. Furthermore, we have examined the association of *G-6-PD* variant and these polymorphisms with severity of neonatal hyperbilirubinemia.

G-6-PD deficiency is well documented as a risk factor for severe neonatal hyperbilirubinemia[25]. The present study confirmed *G-6-PD* variant is associated with more severe hyperbilirubinemia (higher peak TSB than average), longer duration of phototherapy, early onset (≤48 hours) of hyperbilirubinemia, and more hospital readmission. These findings were consistent with previous studies of neonates in northeastern Thailand[20] and with those reported from African American male newborns with G-6-PD deficiency[26] and newborns in Taiwan with G-6-PD deficiency[27]. In the present study, neonates with G-6-PD variants did not reach a statistically significant risk factor for requirement of phototherapy. This might be due to the majority (83.8%) of neonates with hyperbilirubinemia in our setting received phototherapy. Although hemolysis is a major etiologic factor in G-6-PD deficiency-related neonatal hyperbilirubinemia,[28] severity of neonatal hyperbilirubinemia is the result of complex interactions among various mutant genes, especially those involved in bilirubin metabolism, and environmental factors[25].

To the best of our knowledge, the present study is the first to report the presence of *UGT1A1* g.-3279T>G polymorphism in Thai neonates. The allele frequency of *UGT1A1* g.-3279G in newborns with hyperbilirubinemia (40.4% or 0.404) was similar to those reported in Egyptians (0.49),[15] Indians (0.55-0.58)[14, 29] and Malays (0.49)[13]. Although *UGT1A1* g.-3279G allele frequency is significantly higher in neonates with hyperbilirubinemia than that of control neonates, there is no statistically significant association with severity of this condition.

Table 2 Genotype and allele frequencies of *UGT1A1*, *SLCO1B 1*, and *G-6-PD* variants

Gene variant	Number	Genotype frequency (%)	Allele frequency (%)	P_{HWE}
***UGT1A1* g.-3279 T>G**				
T/T	75	36.8		
T/G	93	45.6		
G/G	36	17.6		
T allele	243		59.6	0.51
G allele	165		40.4	
***SLCO1B1* c.388 G>A**				
G/G	125	61.3		
G/A	67	32.8		
A/A	12	5.9		
G allele	317		77.7	0.59
A allele	91		22.3	
***SLCO1B1* c.521 T>C**				
T/T	164	80.4		
T/C	38	18.6		
C/C	2	1.0		
T allele	366		89.7	0.91
C allele	42		10.3	
***G-6-PD* variant**				
Hemizygote	76	37.2	NA	NA
Heterozygote	24	11.8	NA	NA
Homozygote	3	1.5		
Total	103	50.5		

Note: NA, not applicable due to G-6-PD deficiency is an X-linked disorder; P_{HWE}, p-value of Chi-Square test for Hardy-Weinberg equilibrium.

Yusoff *et al*. reported among Malay infants with hyperbilirubinemia there is no significant difference in mean peak TSB or early onset of hyperbilirubinemia among those carrying *UGT1A1* g.-3279T>G of various genotypes[13]. There is a high frequency (50%) of *UGT1A1* g.-3279T>G polymorphism among Indonesian neonates with hyperbilirubinemia but this polymorphism is not associated with severity of hyperbilirubinemia[30]. On the other hand, among Egyptian newborns presenting with jaundice, *UGT1A1* g.-3279T>G polymorphism is significantly associated with higher mean peak TSB, higher bilirubin/albumin ratio, and longer duration of hospital stay[15].

Table 3 Association between genetic variations and continuous variables analyzed by multiple linear regression analysis

Genetic variation	Peak TSB (mg/dL)	Duration of phototherapy (hours)
G-6-PD variant	0.93 (95% CI: 0.22-1.64)	14.45 (95% CI: 6.92-21.99)
UGT1A1 g.-3279T>G	-0.36 (95% CI: -1.10-0.38)	-2.61 (95% CI: -10.48-5.27)
*SLCO1B1*c.388G>A	0.66 (95% CI: -0.52-1.83)	2.45 (95% CI: -9.79-14.69)
SLCO1B1 c.521T>C	-0.25 (95% CI: -1.39-0.90)	-0.28 (95% CI: -12.01-11.45)

Note: Data are presented as coefficient value (95% CI) and statistically significant results (*p*-value < 0.05**)** are indicated in bold text**.**

Table 4 Association between genetic variations and categorical variables analyzed by multiple logistic regression analysis

Genetic variation	Onset of Hyperbilirubinemia ≤48 hr.	Requirement of phototherapy	Hospital readmission
G-6-PD variant	**2.29 (95% CI: 1.22-4.31)**	2.17 (95% CI: 0.92-5.13)	**4.13 (95% CI: 1.09-15.67)**
UGT1A1 g.-3279T>G	0.86 (95% CI: 0.45-1.65)	1.62 (95% CI: 0.71-3.68)	0.39 (95% CI: 0.12-1.22)
SLCO1B1 c.388G>A	1.18 (95% CI: 0.43-3.22)	0.41 (95% CI: 0.08-2.00)	0.15 (95% CI: 0.02-0.85)
SLCO1B1 c.521T>C	0.98 (95% CI: 0.37-2.62)	1.49 (95% CI: 0.30-7.32)	1.96 (95% CI: 0.53-7.25)

Note: Data are presented as odd ratio (95% CI) and statistically significant results (*p*-value < 0.05**)** are indicated in bold text**.**

These controversial results of association between *UGT1A1* g.-3279T>G variant and severity of hyperbilirubinemia can be partly explained by the differences in genetic background and environment among different populations[31].

Genetic variations in *SLCO1B1* have been investigated in different populations. *SLCO1B1* c.388G allele is highly prevalent in Asian populations with allele frequency of 60-90%, but is less frequent in Caucasians, with allele frequency of 30-45%[32]. On the other hand, *SLCO1B1* c.521C allele is less prevalent, being present in Asian populations with allele frequency of 10-15%, in Caucasians with allele frequency of 15-20%, and in African-Americans with allele frequency of 1-2%[32]. The c.521C allele is associated with markedly reduced transport activity, while transport function of c.388G allele shows normal, decreased or increased uptake activity toward various substrates[33]. The roles of *SLCO1B1* c.388G>A and c.521T>C polymorphisms in neonatal hyperbilirubinemia are controversial[31]. This is the first report of *SLCO1B1* c.388G>A and c.521T>C polymorphisms in neonates of northeastern Thailand. Their allele frequencies (c.338G 77.7% and c.521C 10.3%) were in the same range of Asian populations[32]. Statistical analysis showed both *SLCO1B1* c.388G>A and *SLCO1B1* c.521T>C polymorphisms were not genetic risk factors for severe neonatal hyperbilirubinemia, consistent with the previous meta-analysis study[17] and more recent studies conducted in Chinese,[34] Indonesian[35] and Taiwanese[36] neonates. However, an earlier report from India indicated association of both c.388G and c.521C alleles with neonatal hyperbilirubinemia[29]. In addition, Liu *et al.* showed in China *SLCO1B1* c.388A, but not c.521C, allele is associated with neonatal hyperbilirubinemia[24]. Therefore, the role of *SLCO1B1* c.388G>A and c.521T>C polymorphisms on severity of neonatal hyperbilirubinemia remains controversial among different populations or ethnic groups.

The etiology of neonatal hyperbilirubinemia consists of complex multifactorial factors, both genetic and environmental,[31] and focusing on a single factor (genetic or environmental) might be fruitful. Expression of multiple bilirubin metabolism gene variants can contribute to increase risk for severe neonatal hyperbilirubinemia[37].

Conclusion

In summary, the study is the first report of *UGT1A1* g.-3279T>G, *SLCO1B1* c.388G>A and *SLCO1B1* c.521T>C polymorphisms in neonates with hyperbilirubinemia in northeast Thailand; however, their carriage is not significantly associated with severity of this condition. Inheritance of G-6-PD variants is independent risk factor for severe neonatal hyperbilirubinemia. A large cohort study will be necessary to understand more fully the interactions among bilirubin metabolism gene variants, other variants of pertinent gene and environmental factors impacting neonatal hyperbilirubinemia.

Take home messages

- The first report of *UGT1A1* g.-3279T>G, *SLCO1B1* c.388G>A and *SLCO1B1* c.521T>C polymorphisms in northeast Thailand.
- *UGT1A1* and *SLCO1B1* polymorphisms are not associated with severe neonatal hyperbilirubinemia.
- *G-6-PD* variants are independent risk factors for severe neonatal hyperbilirubinemia.

Conflicts of interest

The authors declare no conflict of interest.

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