RELATIONSHIP BETWEEN GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY AND *UGT1A1* GENOTYPES IN NEONATES WITH HYPERBILIRUBINEMIA

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Abstract. Hyperbilirubinemia is one of the most common problems in neonates**.** Homozygosity of substitution/indel in coding and promoter regions of *UGT1A1* (encoding uridine diphosphate glucuronosyl transferase 1A1) have been reported to pose additional risk factors for hyperbilirubinemia in glucose-6-phophate dehydrogenase (G6PD)-deficient neonates. The relationship between these mutations on neonatal hyperbilirubinemia has not been investigated in the Northeast Thailand. *UGT1A1* $TA_{(7)}$ promoter mutation and 211G>A variant were analyzed in 108 G6PD-normal and 111 G6PD-deficient neonates with hyperbilirubinemia. There are significant differences in peak total serum bilirubin level among G6PD-normal and -deficient neonates carrying wild type *UGT1A1* (n = 67 and 53, respectively), 211A/A (n = 1 and 1), $TA_{(7)}/TA_{(7)}$ (n = 1 and 1), 211G/A (n = 12 and 17), and $TA_{(6)}/TA_{(7)}$ (n = 20 and 12). Percent hospital re-admission with hyperbilirubinemia is significantly lower in neonates carrying *UGT1A1* $TA_{(6)}/TA_{(7)}$. Further studies with a larger study population are needed to verify these findings.

Keywords: G6PD deficiency, *UGT1A1*, neonatal hyperbilirubinemia, northeastern Thailand

INTRODUCTION

Hyperbilirubinemia is the most common condition requiring evaluation and treatment in neonates and the most frequent cause for hospital re-admission during the first week of postnatal life (Maisels and Kring, 1998). Prevalence and severity of neonatal hyperbilirubinemia are higher among Asians than Caucasians (Seita *et al*, 2002). Genetic and environmental factors contribute to the development of hyperbilirubinemia and the impact of genetic variations on this condition is increasingly being recognized (Kaplan and Hammerman, 2010).

Association between mutations in *UGT1A1* (encoding uridine diphosphate glucuronosyl transferase 1A1) causing Gilbert syndrome and neonatal hyperbilirubinemia has been recognized (Kaplan *et al*, 1997; Boo *et al*, 2009; Prachukthum *et al*, 2012). Homozygous $(TA)_7$ element in promoter region of *UGT1A1* is associated

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with neonatal hyperbilirubinemia among Caucasians, while both homozygous and heterozygous *UGT1A1* G211A variant are more common among Asians. A case-control study and meta-analysis confirmed the latter's association (Long *et al*, 2011). Interestingly, Kaplan *et al* (1997) were the first to demonstrate a relationship between *UGT1A1* promoter mutation and glucose-6-phosphate dehydrogenase (G6PD) deficiency resulting in enhancement of neonatal hyperbilirubinemia, but not either alone.

The association of G6PD deficiency and neonatal hyperbilirubinemia in Thailand was first reported by Flatz *et al* (1963). Studies conducted in Bangkok demonstrated that among neonates with hyperbilirubinemia the prevalence of G6PD deficiency is 21.2 - 22.1% in males (Nuchprayoon *et al*, 2002; Prachukthum *et al*, 2009). The potential for variants of G6PD and of mutant *UGT1A1* to modulate neonatal hyperbilirubinemia is increasingly being recognized (Kaplan *et al*, 1997; Huang *et al*, 2002). However, in Thailand there is no well-documented report on the contribution of G6PD deficiency and *UGT1A1* mutations to the development of neonatal hyperbilirubinemia.

Thus, we studied the association of UGT1A1 A211 variant and of $(TA)_7$ promoter element and hyperbilirubinemia in G6PD-normal and -deficient neonates to determine whether such associations existed and how they impacted on neonatal hyperbilirubinemia in northeastern Thailand.

MATERIALS AND METHODS

Study subjects and sample analysis

Unused EDTA blood samples of neonates with hyperbilirubinemia collected from routine screening for G6PD deficiency at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand between August, 2012 and August, 2014 were used. Classification into G6PD normal ($n = 108$) and deficiency ($n = 111$) was based on a fluorescent spot test (Beutler, 1966). Neonatal hyperbilirubinemia is defined as having TSB level exceeding the 95th percentile according to Bhutani nomogram (Bhutani *et al*, 1999). We excluded neonates who had major congenital anomalies, infection requiring antibiotic treatment, significant hemorrhage from cephalhematoma and whose mothers had diabetes. Neonates with hyperbilirubinemia were treated following the 2004 American Academy of Pediatrics (AAP) guideline (AAP, 2004).

The study was approved by the Institutional Review Board of Khon Kaen University (HE 551273).

Analysis of *UGT1A1*

Total genomic DNA was isolated from whole blood sample using DNAzol reagent (Invitrogen, Carlsbad, CA). *UGT1A1* G211A mutation was detected using RFLP-PCR as describe previously (Huang *et al*, 2004). In brief, the primers used were 211 F (5'-AGATACTGTTGATCCCAGTG-3') and 211 R (5'-CTTCAAGGTGTAAAATGGTC-3'). PCR was performed in a 25-μl reaction mixture containing PCR buffer (100 mM Tris–HCl, 500 mM KCl, 15 mM $MgCl_2$, and 0.01% gelatin), 1.0 U *Taq* DNA polymerase (Invitrogen), 20 ng of each primer, 200 μM dNTPs and 50 ng DNA templates. Thermocycling (conducted in TProfessional thermal cycler; Biometra, Gottingen, Germany) conditions were as follows: 94°C for 5 minutes; 35 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C; and a final step for 10 minutes at 72°C. Amplicon was digested with *Ava*II (New England Biolabs, Ipswich, MA,) and separated by 3% agarose

gel-electrophoresis, stained with ethidium bromide and visualize under UV-light. Amplicons of 18 and 128 bp were obtained for G211and A211 generated a 146-bp fragment. *UGT1A1* $TA_{(n)}$ promoter element was detected using PCR as describe previously (Monaghan *et al*,1996). In short, the primers used were TAF (5'-GTCACGTGACACAGTCAAAC-3') and TAR (5'-TTTGCTCCTGCCAGAGGTT-3' to generate a 98-bp ($TA_{(6)}$) or 100-bp ($TA_{(7)}$) amplicon. PCR reaction was carried out in a 25-µl reaction containing PCR buffer (100 mM Tris–HCl, 500 mM KCl, 15 mM $MgCl_2$, and 0.1% gelatin), 1 U *Taq* DNA polymerase (Invitrogen), 20 ng of each primer, 200 µM dNTPs and 50 ng of DNA template. PCR thermocycling (conducted in TProfessional thermal cycler; Biometra) conditions were as follows: at 95°C for 5 minutes; 30 cycles of 95°C for 5 minutes, 58°C for 40 seconds, and 72°C for 40 seconds; and a final step for 10 minutes at 72°C. Amplicons were separated by 10% polyacrylamide gel-electrophoresis, stained with ethidium bromide and visualize under UV-light.

Statistical analysis

Statistical analysis was performed using Minitab statistical software version 14 (Minitab, State College, PA). Descriptive statistics, mean and standard deviation (SD) are applied to each group. Student's *t*-test or Mann-Whitney *U* test was used to test significant difference between two independent continuous variables, and proportion or Fisher's exact test between two independent categorized variables. A statistically significant difference is accepted at a p-value < 0.05.

RESULTS

There are no statistical significant difference in birth weight, gestational age, delivery method, type of feeding, excessive weight loss, need of blood exchange transfusion, and rate of re-admission with hyperbilirubinemia between hyperbilirubinemic neonates with and without G6PD deficiency (Table 1). However, G6PD-deficient neonates constitute a significantly higher number of males, those requiring phototherapy and those with higher mean peak total serum bilirubin (TSB).

Based on samples with complete *UGT1A1* genotyping data, there was one case each of G6PD-normal with homozygous A211, G6PD-normal with homozygous $TA_{(7)}$ sequence, G6PD-deficiency with homozygous A211, and G6PD-deficiency with homozygous $TA_{(7)}$; 12 and 17 cases of G6PD-normal and -deficiency with heterozygous A211, respectively; and 20 and 12 cases of G6PD-normal and -deficiency with heterozygous $TA_{(7)}$, respectively (PCR data not shown). All the remaining neonates (G6PD-normal = 67, G6PD-deficient = 53) carried normal *UGT1A1*. When mean peak TSB levels were compared, that of G6PD-deficient neonates carrying wild type *UGT1A1* is significantly higher than of G6PD-normal neonates, and, similarly, that of G6PD-normal neonates carrying 211G/A to those carrying wild type (Table 2). It is worth noting that among the two cases each of 211A/A and $TA_{(7)}/TA_{(7)}$, peak TSB levels of G6PD-deficient neonates were higher than of G6PD- normal, and peak TSB level of neonates the former *UGT1A1* genotype was higher than of the latter in the same G6PD status group.

Although the proportion of hospital re-admission within 30 days with hyperbilirubinemia is not significantly different in both G6PD-normal and -deficient neonates, those carrying *UGT1A1* $TA_{(6)}/TA_{(7)}$ were least prone compared to those carrying wild type gene (Table 3).

Table 1
Demographic characteristics of glucose-6-phosphate dehydrogenase (G6PD)-normal and -deficient neonates with hyperbilirubinemia.

Characteristic	G6PD-normal (n = 108)	G6PD-deficient (n = 111)	p-value*
Birth weight (g)	3,117 ± 418[a]	3,076 ± 472	NS
Gestational age (week)	38.0 ± 1.3	38.2 ± 1.3	NS
Male, n (%)	36 (33)	97 (87)	0.0001
Delivery			
Normal, n (%)	51 (49)	49 (44)	NS
Cesarean section, n (%)	47 (46)	59 (53)	NS
Forceps or vacuum extraction, n (%)	5 (5)	3 (3)	NS
Type of feeding			
Exclusive breast-feeding, n (%)	108 (100)	110 (99)	NS
Infant formula, n (%)	0 (0)	1 (1)	NS
Peak TSB (mg/dl)	14 ± 2	16 ± 3	0.001
Excessive weight loss (>10%), n (%)	16 (15)	11 (10)[b]	NS
Phototherapy, n (%)	91 (85)[c]	110 (99)	0.0001
Blood exchange transfusion, n (%)	0 (0)	1 (1)	NS
Re-admission with hyperbilirubinemia[d], n (%)	7 (7)	13 (12)	NS

NS, not significant; TSB, total serum bilirubin. [a]Mean ± SD. [b]Total n = 110. [c]Total n = 107. [d]Within 30 days. *Significant at p-value < 0.05.

DISCUSSION

Homozygosity of *UGT1A1* mutations were reported to be associated with more severe hyperbilirubinemia in neonates with G6PD deficiency (Kaplan *et al*, 1997; Huang *et al,* 2002). In our study, among the two cases with homozygous UGT1A1 A211 variant or $TA_{(7)}$ promoter mutation, hyperbilirubinemic neonates with G6PD deficiency had higher peak TSB level. Boo *et al* (2009) showed that homozygosity of A211 mutation in 27 G6PD-deficient Malaysian neonates is a significant risk factor in the development of severe hyperbilirubinemia. However, Sato *et al* (2013) found in Japan a significant increased peak bilirubin level associated with incidence of hyperbilirubinemia in 127 neonates with heterozygous UGT1A1 A211 variant but not with homozygous $TA_{(7)}$ mutation, and the presence of 211A/A is only a risk factor for neonatal hyperbilirubinemia among 11 Taiwanese G6PD-deficient hemizygote neonates (Huang *et al,* 2002). Interestingly, an association between UGT1A1 A211 variant and neonatal hyperbilirubinemia was reported in Thailand (Prachukthum *et al*, 2012). Differences among these studies may reflect differences in prevalence of various mutant *UGT1A1* genotypes and sample size of the populations studied.

Mean peak TSB in G6PD-deficient neonates with wild type *UGT1A1* is significantly higher than those with normal G6PD, similar to previous reports (Riskin *et al,* 2012; Badejoko *et al,* 2014). Although rate of hospital re-admission due to re-occurrence of hyperbilirubinemia was low,

Table 2
Peak total serum bilirubin (TSB) level of glucose-6-phosphate dehydrogenase (G6PD)-normal and -deficient neonates with hyperbilirubinemia carrying various *UGT1A1* genotypes.

UGT1A1 genotype	Peak TSB (mg/dl)	
	G6PD-normal Mean ± SD (*n*)	G6PD-deficient Mean ± SD (*n*)
211G/G, $TA_{(6)}/TA_{(6)}$ (wild type)	14 ± 2 (67)	15 ± 3 (53)[a]
211A/A, $TA_{(6)}/TA_{(6)}$	12 (1)	33 (1)
211G/G, $TA_{(7)}/TA_{(7)}$	8 (1)	18 (1)
211G/G, $TA_{(6)}/TA_{(7)}$	15 ± 3 (20)	15 ± 3 (12)
211G/A, $TA_{(6)}/TA_{(6)}$	15 ± 2 (12)[b]	16 ± 3 (17)

[a]$p = 0.002$, compared with G6PD normal. [b]$p = 0.032$, compared with wild type *UGT1A1*. Significant at a p-value < 0.05. Total number of samples is < than that in Table 1 as only those with complete genotype are included.

Table 3
Hospital re-admission rate with hyperbilirubinemia of glucose-6-phosphate dehydrogenase (G6PD)-normal and -deficient neonates with same previous syndrome carrying various *UGT1A1* genotypes.

UGT1A1 genotype	Hospital re-admission[a]	
	G6PD-normal *n*/total *n* (%)	G6PD-deficient *n*/total *n* (%)
211G/G, $TA_{(6)}/TA_{(6)}$ (wild type)	4/67 (6)	8/53 (15)
211A/A, $TA_{(6)}/TA_{(6)}$	0/1 (0)	1/1 (100)
211G/G, $TA_{(7)}/TA_{(7)}$	0/1 (0)	0/1 (0)
211G/A, $TA_{(6)}/TA_{(6)}$	2/12 (17)	2/17 (12)
211G/G, $TA_{(6)}/TA_{(7)}$	0/20 (0)[b]	0/12 (0)[c]

[a]Within 30 days. [b]$p = 0.039$, compared to wild type *UGT1A1*. [c]$p = 0.002$, compared to wild type *UGT1A1*. Significant at a p-value < 0.05. Samples are those listed in Table 2.

it is worth noting that neonates carrying *UGT1A1* $TA_{(6)}/TA_{(7)}$ had minimal risk of re-admission. Future studies involving a larger cohort should indicate if such genetic markers can be of prognostic value.

In summary, this study could not clearly demonstrate the association of homozygosity of UGT1A1 A211 variant or of $TA_{(7)}$ promoter mutation with higher peak total serum bilirubin level in G6PD-deficient neonates with hyperbilirubinemia, but did show an association of reduced risk of hospital re-admission due to relapse of hyperbilirubinemia in neonates with certain *UGT1A1* genotype. Further studies should be carried out on a larger study populations to verify these findings.

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