วารสาร
เทคนิคการแพทย์และกายภาพบำบัด
JOURNAL OF MEDICAL TECHNOLOGY AND PHYSICAL THERAPY

นิพนธ์ต้นฉบับ
ORIGINAL ARTICLE

ความสัมพันธ์ระหว่างการกลายพันธ์ของยีน G6PD โพลิมอร์ฟิซึมของยีน HMOX1 และโพลิมอร์ฟิซึมของยีน BLVRA กับความรุนแรงของภาวะตัวเหลือง ในทารกแรกเกิดชาวไทย ในภาคตะวันออกเฉียงเหนือ

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Received: *September 24, 2019*
Revised: *December 3, 2019*
Accepted: *January 2, 2020*

บทคัดย่อ

ภาวะตัวเหลืองในทารกแรกเกิดเป็นภาวะที่พบบ่อยในทารกแรกเกิด ซึ่งเกี่ยวข้องกับหลายปัจจัย โดยเฉพาะพันธุกรรม ในการศึกษานี้จึงมีวัตถุประสงค์เพื่อหาความชุกของการกลายพันธุ์ของยีน *G6PD*, โพลิมอร์ฟิซึมของยีน *HMOX1* (GT- repeats) และ *BLVRA* (rs699512; A>G) และศึกษาความสัมพันธ์ของการมีโพลิมอร์ฟิซึมเหล่านี้ต่อความรุนแรงของภาวะตัวเหลืองในทารกแรกเกิด โดยศึกษาในตัวอย่างทารกแรกเกิดที่มีภาวะตัวเหลืองทั้งหมด 271 รายซึ่งทำการตรวจกลายพันธุ์ของยีน *G6PD* และโพลิมอร์ฟิซึมของยีน *BLVRA* (rs699512; A>G) ด้วยวิธี AS-PCR และ PCR-RFLP ตามลำดับ ส่วนการตรวจโพลิมอร์ฟิซึม GT-repeats ของยีน *HMOX1* ใช้เทคนิค PCR และ fragment analysis พบความชุกของการกลายพันธุ์ของยีน *G6PD* โพลิมอร์ฟิซึม*HMOX1* (≤23 GT repeats) และ *BLVRA* (rs699512; A>G) ร้อยละ 55.7, 62.3 และ 42.1 ตามลำดับ เมื่อวิเคราะห์ความสัมพันธ์ระหว่างโพลิมอร์ฟิซึมของยีนต่างๆ กับระดับความรุนแรงของภาวะตัวเหลืองในทารกแรกเกิด พบว่าการกลายพันธุ์ของยีน *G6PD* และการพบโพลิมอร์ฟิซึมชนิด *HMOX1* (≤ 23 GT repeats) และ *BLVRA* (rs699512; A>G) เป็นปัจจัยเสี่ยงที่สำคัญของการเกิดภาวะตัวเหลืองที่รุนแรงมากขึ้นในทารกแรกเกิด ดังนั้นในทารกแรกเกิดที่มีภาวะตัวเหลืองรุนแรง จึงควรพิจารณาตรวจหาการกลายพันธุ์ของยีน *G6PD* หรือโพลิมอร์ฟิซึมชนิด *HMOX1* (≤ 23 GT repeats) หรือ *BLVRA* (rs699512; A>G) ร่วมด้วย

คำสำคัญ: ภาวะตัวเหลืองในทารกแรกเกิด, *G6PD, HMOX1, BLVRA*

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ORIGINAL ARTICLE

Association between G6PD, HMOX1 and BLVRA variants and severity of hyperbilirubinemia in northeastern Thai neonates

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Abstract

Neonatal hyperbilirubinemia is a common disorder that is associated with a variety of factors, especially genetic factors. This study aimed to examine the prevalence of *G6PD, HMOX1* (GT-repeats) and *BLVRA* (rs699512; A>G) variants and their association with the severity of neonatal hyperbilirubinemia in northeastern Thai neonates. A total of 271 neonates with hyperbilirubinemia were analyzed for *G6PD* mutation, *HMOX1* (GT-repeats) and *BLVRA* (rs699512; A>G) polymorphisms by multiplex AS-PCR, fragment analysis and PCR-RFLP, respectively. The prevalence of *G6PD* mutation, *HMOX1* (short GT repeats) and *BLVRA* (rs699512; A>G) polymorphisms were found in 55.7%, 62.3% and 42.1% of subjects, respectively. The present study showed that the *G6PD* mutation, *HMOX1* (≤ 23 GT-repeats) and *BLVRA* (rs699512; A>G) polymorphisms were significant risk factors for developing severe neonatal hyperbilirubinemia. Therefore, neonates with severe hyperbilirubinemia should be investigated for the *G6PD* mutation or *HMOX1* (≤ 23 GT-repeats) or *BLVRA* (rs699512; A>G) polymorphisms.

Keywords: Neonatal hyperbilirubinemia, *G6PD, HMOX1, BLVRA*

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Introduction

Hyperbilirubinemia is the most common condition found in neonates. It is a multifactorial disorder involving bilirubin over-production, reduced conjugation and impaired bilirubin excretion[1]. Common risk factors for neonatal hyperbilirubinemia consist of fetal-maternal blood group incompatibility, breastfeeding, prematurity and G6PD deficiency[2]. Moreover, in recent years, the role of genetic factors in the bilirubin metabolism pathway has become apparent[3].

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme that catalyzes the first step in the pentose phosphate pathway. It is essential for maintaining a normal red blood cell's life span and for oxidizing processes[4]. G6PD deficiency is a risk factor for severe hyperbilirubinemia and this deficiency is caused by mutations in the *G6PD* gene[5]. Previous studies have shown that G6PD Viangchan (871G>A), G6PD Mahidol (487G>A), G6PD Union (1360C>T), G6PD Canton (1376G>T), G6PD Kaiping (1388G>A), G6PD Chinese-4 (392G>A) and G6PD Chinese-5 (1024C>T) are the common variants in Thailand[6].

Heme oxygenase 1 (HMOX1) is the initial and rate-limiting enzyme in the conversion of heme to biliverdin, which is further metabolized to bilirubin[7]. The (GT)n dinucleotide length polymorphism of the *HMOX1* promoter may modulate the transcriptional activity (gene expression); increased HMOX1 activity will result in an overproduction of bilirubin[8].

Biliverdin reductase A (BLVRA) is an enzyme that reduces biliverdin to bilirubin[9]. In the *BLVRA* gene, there is rs699512; A>G polymorphism, the only common non-synonymous SNP within the BLVRA gene, as shown in the dbSNP database. It is possible that SNPs within the *BLVRA* gene might affect TSB levels [10].

In this study, we determined the prevalence of *G6PD*, *HMOX1* (GT-repeats) and *BLVRA* (rs699512; A>G) variants and the association of these variants with the severity of hyperbilirubinemia in northeastern Thai neonates with hyperbilirubinemia.

Materials and methods

Subjects

Ethical approval for the study protocol was obtained from the Institutional Review Board (IRB) of Khon Kaen University, Khon Kaen, Thailand (HE611463). Based on the prevalence of these polymorphisms reported in previous studies [11-13], the sample size required was statistically estimated at 271. One hundred and forty DNA samples were recruited from a previous study (HE591531)[14] and one hundred and thirty-one leftover EDTA blood samples of neonates with hyperbilirubinemia were collected from routine screening for G6PD deficiency using fluorescent spot test (FST) at the Diagnostic Microscopy Unit, Clinical Laboratory Division, Srinagarind Hospital, Khon Kaen University.

Criteria for study subjects

The subjects included near full-term and full-term neonates (35-42 weeks of gestation) with hyperbilirubinemia (hyperbilirubinemia was defined by the American Academy of Pediatrics 2004) [15]. Neonates with known risk factors for neonatal hyperbilirubinemia were excluded, including maternal history with diabetes mellitus, neonates with ABO and Rh incompatibility, neonates with infection requiring antibiotics and neonates with congenital anomalies.

Clinical data were acquired from medical records by a pediatrician. The clinical data collected in this study included maximum total serum bilirubin level, the onset of hyperbilirubinemia, requirement for phototherapy, duration of phototherapy and hospital re-admission.

Molecular analysis

Genomic DNA was isolated from peripheral blood leukocytes. The DNA was extracted from leukocytes using a DNAzol kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. All DNA samples were screened for seven *G6PD* mutations commonly present in Thai populations, including G6PD Viangchan (871G>A), G6PD Mahidol (487G>A), G6PD Union (1360C>T), G6PD Canton (1376G>T), G6PD Kaiping (1388G>A), G6PD Chinese-4 (392G>A) and G6PD Chinese-5 (1024C>T) by multiplex allele-specific polymerase chain reaction (AS-PCR) as previously described[16]. AS-PCR positive samples were selected for zygosity detection by PCR-RFLP, as previously described[6].

The *HMOX1* (GT-repeats) polymorphism was identified by fragment analysis. The primers FF-127: 5'FAM-AGAGCCTGCAGCTTCTCAGA-3' and R-127: 5'-ACAAAGTCTGGCCATAGG-AC-3' were used in the polymerase chain reaction (PCR). The lengths of the PCR products were determined on a fragment analyzer using Peak Scanner v1.0 (Applied Biosystems, Foster City, CA, USA) on the ABI3730xl sequencer with Peak Scanner V.1.0 software-500 LIZ (Applied Biosystems) as a size marker.

The *BLVRA* (rs699512; A>G) polymorphism was detected by the PCR-RFLP method with a mismatched primer set (F-BV512: 5'-GACCT-GAACCTCTGCTTTTG-3' and R-BV512: 5'- TACAAA-GACCAGTTTAAGGGATG-3') and HpyCH4V restriction enzymes (New England Biolabs, Ipswich, MA, USA). The samples that were identified as wild type, heterozygote and homozygote by the PCR-RFLP method were selected to be confirmed by DNA sequencing.

Statistical analysis

Statistical analysis was performed using STATA software version 10.1 (StataCorp LLC, Texas, USA). Prevalence and allele frequency of each polymorphism were presented as percentage and 95% confident interval (CI). The association between the categorical outcome variables (including the onset of hyperbilirubinemia ≤48 hr., phototherapy and hospital re-admission) and the gene polymorphisms were assessed using multiple logistic regression analysis. For continuous outcomes (including maximum TSB and duration of phototherapy), a multiple linear regression analysis was applied. The Odds ratio and coefficient value with 95% confident interval (CI) were presented to determine the significance of the association. A *p-value* < 0.05 was considered to indicate statistical significance.

Results

The prevalence of *G6PD* mutation, *HMOX1* (GT-repeats) polymorphism and *BLVRA* (rs699512; A>G) polymorphism.

A total of 271 neonates with hyperbilirubinemia were included in this study. The prevalence of *G6PD* mutation, *HMOX1* (GT-repeats) polymorphism and *BLVRA* (rs699512; A>G)

polymorphism are shown in **Table 1**. The *HMOX1* (GT-repeats) and *BLVRA* (rs699512; A>G) genotype frequencies were consistent with Hardy-Weinberg equilibrium (p = 0.27 and p = 0.69, respectively). The *G6PD* mutations were identified in 151 samples (55.7%). Heterozygotes, homozygotes and hemizygotes were identified in 34 samples (12.5%), 9 samples (3.3%) and 108 samples (39.9%), respectively.

The numbers of GT-repeats of the *HMOX1* promoter were highly polymorphic, ranging from 15 to 40. The two most frequent alleles had 23 and 30 GT-repeats (**Figure 1**). The alleles were grouped into two subclasses; short (S) allele with ≤23 GT-repeats and long (L) allele with >23 GT-repeats. The L/L genotype was found in 102 samples (37.7%), the S/L genotype was found in 99 samples (36.5%), and the S/S genotype was found in 70 samples (25.8%). The frequencies of the L and S alleles were 0.56 and 0.44, respectively.

The *BLVRA* rs699512 A>G polymorphism was found in 114 samples (42.1%). Heterozygotes (A/G) and homozygotes (G/G) were identified in 101 samples (37.3%) and 13 samples (4.8%), respectively. The frequency of A and T alleles were 0.77 and 0.23, respectively.

Associations of *G6PD* mutation, *HMOX1* (GT-repeats) polymorphism and *BLVRA* (rs699512; A>G) polymorphism with severity of hyperbilirubinemia

A multiple regression analysis was applied to determine the association between the gene polymorphisms and the severity of hyperbilirubinemia in neonates, using the *G6PD* mutation, *HMOX1* (≤23 GT-repeats) polymorphism, and *BLVRA* (rs699512; A>G) polymorphism as independent variables. The maximum TSB, the onset of hyperbilirubinemia ≤48 hr., phototherapy, duration of phototherapy and hospital re-admission were dependent variables. The association of the genetic variables and severity for neonatal hyperbilirubinemia are shown in **Table 2** and **Table 3**. The *G6PD* mutation was found to be significant independent predictors for maximum TSB (coefficient 0.62, 95% CI: 0.02-1.22, *p-value* 0.043) and duration of phototherapy (coefficient 10.32, 95% CI: 4.72-15.93, *p-value* 0.000). Furthermore, The *G6PD* mutation was also a significant independent risk factor for onset of hyperbilirubinemia≤48 hr (OR 2.37, 95% CI: 1.42-3.98, *p-value* 0.001), requirement for phototherapy (OR 2.17, 95% CI: 1.10-4.23, *p-value* 0.024), and hospital re-admission (OR 2.77, 95% CI: 1.06-7.24, *p-value* 0.038).

The *HMOX1* (≤23 GT-repeats) polymorphism was found to be a significant independent predictor for maximum TSB (coefficient 0.48, 95% CI: 0.01-1.04, *p-value* 0.037).

The *BLVRA* (rs699512; A>G) polymorphism was found to be a significant independent predictor variable for the duration of the phototherapy (coefficient 8.03, 95% CI: 2.46-13.61, *p-value* 0.005) and it was found to be a significant independent risk factor for hospital re-admission (OR 2.44, 95% CI: 1.04-5.68, *p-value* 0.039).

Table 1 Prevalence of *HMOX1* (GT-repeats), *HMOX1* (-413A>T) and *BLVRA* (rs699512; A>G) polymorphisms and proportion of various *G6PD* genotypes (n = 271)

Polymorphism	Genotype frequency, Number of subjects (%)	Allele frequency	P_{HWE}
HMOX1			
(GT)n repeats[a]			0.27
L/L	102 (37.7)		
S/L	99 (36.5)		
S/S	70 (25.8)		
L allele		0.56	
S allele		0.44	
BLVRA (rs699512; A>G)			0.69
A/A	157 (57.9)		
A/G	101 (37.3)		
G/G	13 (4.8)	0.77	
A allele		0.23	
G allele			
G6PD mutation[b]			NA
No mutation	120 (44.3)		
Heterozygote	34 (12.5)		
Homozygote	9 (3.3)		
Hemizygote	108 (39.9)		

[a] L > 23 repeats, S ≤ 23 repeats

[b] *G6PD* mutations investigated in this study included G6PD Viangchan (871G>A), G6PD Mahidol (487G>A), G6PD Union (1360C>T), G6PD Canton (1376G>T), G6PD Kaiping (1388G>A), G6PD Chinese-4 (392G>A) and G6PD Chinese-5 (1024C>T). NA: not applicable

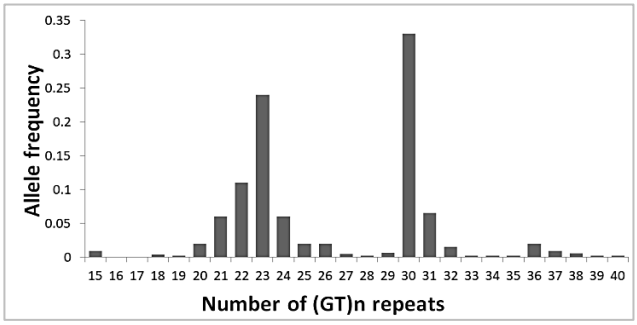

Figure 1 Distribution of the (GT)n repeats of *HMOX1* promoter among 271 neonates. GT-repeats ranged from 15 to 40. Two most frequent alleles were 23 and 30 GT-repeats.

Associations of *G6PD* mutation, *HMOX1* (GT-repeats) polymorphism and *BLVRA* (rs699512; A>G) polymorphism with severity of hyperbilirubinemia

A multiple regression analysis was applied to determine the association between the gene polymorphisms and the severity of hyperbilirubinemia in neonates, using the *G6PD* mutation, *HMOX1* (≤23 GT-repeats) polymorphism, and *BLVRA* (rs699512; A>G) polymorphism as independent variables. The maximum TSB, the onset of hyperbilirubinemia ≤48 hr., phototherapy, duration of phototherapy and hospital re-admission were dependent variables. The association of the genetic variables and severity for neonatal hyperbilirubinemia are shown in **Table 2** and **Table 3**. The *G6PD* mutation was found to be significant independent predictors for maximum TSB (coefficient 0.62, 95% CI: 0.02-1.22, *p-value* 0.043) and duration of phototherapy (coefficient 10.32, 95% CI: 4.72-15.93, *p-value* 0.000). Furthermore, The *G6PD* mutation was also a significant independent risk factor for onset of hyperbilirubinemia≤48 hr (OR 2.37, 95% CI: 1.42-3.98, *p-value* 0.001), requirement for phototherapy (OR 2.17, 95% CI: 1.10-4.23, *p-value* 0.024), and hospital re-admission (OR 2.77, 95% CI: 1.06-7.24, *p-value* 0.038).

The *HMOX1* (≤23 GT-repeats) polymorphism was found to be a significant independent predictor for maximum TSB (coefficient 0.48, 95% CI: 0.01-1.04, *p-value* 0.037).

The *BLVRA* (rs699512; A>G) polymorphism was found to be a significant independent predictor variable for the duration of the phototherapy (coefficient 8.03, 95% CI: 2.46-13.61, *p-value* 0.005) and it was found to be a significant independent risk factor for hospital re-admission (OR 2.44, 95% CI: 1.04-5.68, *p-value* 0.039).

Table 2 Results of multiple linear regression for the association of genetic polymorphisms and severity for neonatal hyperbilirubinemia among 271 neonates with hyperbilirubinemia; presented as coefficient value with 95% confident interval (CI)

Genetic variable	Maximum TSB (mg/dl)	Duration of phototherapy (hr.)
G6PD mutation	**0.62 (95% CI: 0.02, 1.22)** [a]	**10.17 (95% CI: 4.65-15.70)** [a]
HMOX1 (≤23 GT-repeats)	**0.48 (95% CI: 0.01, 1.04)** [a]	-5.69 (95% CI: -11.54, -0.16)
BLVRA (rs699512; A>G)	0.52 (95% CI: -0.55, 0.66)	**8.03 (95% CI: 2.46-13.61)** [a]

[a] Statistically significant; $p < 0.05$

TSB: Total serum bilirubin

Table 3 Results of multiple logistic regression for the association of genetic polymorphisms and severity for neonatal hyperbilirubinemia among 271 neonates with hyperbilirubinemia; presented as Odds ratio value with 95% confident interval (CI)

Genetic variable	Onset of Hyperbilirubinemia ≤48 hr.	Phototherapy	Hospital re-admission
G6PD mutation	**2.37 (95% CI: 1.42-3.98)** [a]	**2.17 (95% CI: 1.10-4.23)** [a]	**2.77 (95% CI: 1.06-7.24)** [a]
HMOX1 (≤23 GT-repeats)	1.07 (95% CI: 0.62-1.83)	0.92 (95% CI: 0.45-1.87)	0.73 (95% CI: 0.35-1.77)
BLVRA (rs699512; A>G)	1.49 (95% CI: 0.89-2.48)	1.18 (95% CI: 0.59-2.32)	**2.44 (95% CI: 1.04-5.68)** [a]

[a] Statistically significant ($p < 0.05$)

Discussion

The present study showed that *G6PD* mutation, *HMOX1* (≤23 GT-repeats) polymorphism and *BLVRA* (rs699512; A>G) polymorphism were considered to be significant risk factors and predictors for the development of neonatal hyperbilirubinemia. To our knowledge, this study is the first report of the prevalence of *HMOX1* (GT-repeats) and *BLVRA* (rs699512; A>G) polymorphisms in Thai neonates.

The G6PD deficiency is widely recognized as a risk factor associated with neonatal hyperbilirubinemia[17-20]. The present study confirmed that *G6PD* mutation was an independent risk factor for all parameters indicating severe hyperbilirubinemia (the onset of hyperbilirubinemia ≤48 hr., higher maximum TSB, requirement for phototherapy, longer duration of phototherapy, and hospital re-admission. The results were

similar to the study of Huang *et al*.[19], who reported that the duration of phototherapy was found to be longer in G6PD deficient male neonates than in the control group. Similarly, Khemtonglang *et al*.[14] reported a longer duration for phototherapy and a higher number of hospital re-admissions in northeastern Thai neonates with G6PD deficiency. Kaplan *et al*.[21] have shown that jaundice in G6PD deficient neonates is the result of an imbalance between the production and conjugation of bilirubin with a tendency for inefficient bilirubin conjugation. Therefore, neonates carrying the G6PD mutation are susceptible to severe hyperbilirubinemia due to an increase in the unconjugated bilirubin. This suggested that the *G6PD* mutation is an important genetic factor associated with severe neonatal hyperbilirubinemia. Thus, an investigation of the *G6PD* mutation is recommended in northeastern Thai neonates with hyperbilirubinemia.

In this study, the (GT)n repeats were found to be highly polymorphic in nature, ranging from 15 to 40. This is consistent with other studies[11, 12, 22]. The study identified the two most frequent alleles, which were 23 and 30 GT-repeats, and is consistent with previous studies from Japan[8, 23, 24] and northern India[11]. In our study, the alleles were grouped into two subclasses; short (S) allele (≤23 GT-repeats) and long (L) allele (>23 GT-repeats). The number of GT repeats promoter has a controlling effect on the rate of gene expression (transcriptional activity). Short allele GT repeats on the promoter may play an important genetic role in up-regulating the equilibrium between bilirubin production and elimination[25]. The result of this study showed that the *HMOX1* short GT-repeats (≤ 23) polymorphism was an independent predictor variable for the higher maximum TSB (coefficient 0.48, 95% CI: 0.01-1.04, *p-value* 0.037). Previous studies showed an association between short GT-repeats and neonatal hyperbilirubinemia. Katayama et al.[8] reported an association between the short allele (< 22 GT-repeats) and hyperbilirubinemia in the early neonatal period in Japanese neonates (OR 3.1, 95% CI: 1.03-9.53). Furthermore, the SS or SL genotypes were more frequent in hyperbilirubinemia neonates than the control group. Their result was similar to those reported by Weng *et al*.[13] and Tiwari *et al*[11]. Weng *et al*.[13] showed that short GT-repeats (< 24 GT-repeats) were highly prevalent in neonatal hyperbilirubinemia. Tiwari *et al*.[11] demonstrated that the SS genotype (S <22 GT-repeats) was significantly associated with neonatal hyperbilirubinemia (OR 4.4, 95% CI: 1.2-16.8). The result of our study showed that the short GT-repeats associated with the higher maximum TSB indicated more severity of neonatal hyperbilirubinemia.

BLVRA is the enzyme that reduces biliverdin to bilirubin in the heme degradation pathway[10]. The result of this study revealed that the *BLVRA* (rs699512; A>G) polymorphism was an independent predictor variable for a longer duration of phototherapy and hospital re-admission. Whereas no association was found between the *BLVRA* (rs699512; A>G) polymorphism and maximum TSB, the onset of hyperbilirubinemia (≤ 48 hrs) and phototherapy. The results are similar to the study of Yang *et al*.[12] which described no correlation between the *BLVRA* (rs699512; A>G) polymorphism and TSB levels in Chinese neonates. Whereas, a recent study[5] showed that the G allele carriers were protected from the development of neonatal hyperbilirubinemia (adjusted OR = 0.86, *p-value*

= 0.04). However, the exact mechanism by which the variation at the rs699512 locus alters the BLVRA function is still unclear. Nevertheless, the present study showed that the *BLVRA* (rs699512; A>G) polymorphism associated with the increased severity of neonatal hyperbilirubinemia.

This study has some limitations. Firstly, this study did not determine the other related genes that are associated with bilirubin metabolism, especially *UGT1A1* polymorphisms, since a previous study in northeastern Thai neonates with hyperbilirubinemia[14] has shown the low allele frequency of the two most common polymorphisms of the *UGT1A1* gene; the frequency of the 211G>A allele was 0.094 and that of TA_7 in promoter was 0.103. Secondly, severe cases of neonatal hyperbilirubinemia did not occur in this study because of a systematic approach for early diagnosis and management of neonates with hyperbilirubinemia at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. Further studies should be carried out to verify the association of all genes involved in bilirubin metabolism to the severity of the hyperbilirubinemia in larger study populations.

Conclusions

The present study showed that the *HMOX1* (≤23 GT-repeats) polymorphism, *BLVRA* (rs699512; A>G) polymorphism and *G6PD* mutation could be considered to be significant risk factors and significant predictors for the development of severe neonatal hyperbilirubinemia. Therefore, in cases of severe neonatal hyperbilirubinemia with unidentified etiology, an investigation for common *G6PD* mutations together with *HMOX1* (≤23 GT-repeats) and/or *BLVRA* (rs699512; A>G) polymorphism should be considered.

Acknowledgements

This work was supported by research grants from Khon Kaen University and the National Research Council of Thailand (NRCT). The authors are grateful to the participants who gave their time in this study.

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