รายงานวิจัยฉบับสมบูรณ์

ภาวะตัวเหลืองในทารกแรกเกิดในภาคเหนือของประเทศไทย: บทบาทของภาวะพร่องเอนไซม์ จี-ซิก-พีดี และอัลฟ่า-ธาลัสซีเมีย

Neonatal hyperbilirubinemia in northern Thailand: roles of G-6-PD deficiency and alpha-thalassemia

โดย

คณะผู้วิจัย	สังกัด
1. รองศาสตราจารย์แพทย์หญิงพิมพ์ลักษณ์ เจริญขวัญ	มหาวิทยาลัยเชียงใหม่
2. ผู้ช่วยศาสตราจารย์แพทย์หญิงวัชรี ตันติประภา	มหาวิทยาลัยเชียงใหม่
3. รองศาสตราจารย์แพทย์หญิงสุพัตรา ศิริโชติยะกุล	มหาวิทยาลัยเชียงใหม่
4. อาจารย์นายแพทย์รวี ทวีผล	มหาวิทยาลัยเชียงใหม่
5. ศาสตราจารย์นายแพทย์ต่อพงศ์ สงวนเสริมศรี	มหาวิทยาลัยเชียงใหม่

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สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษา
และสำนักงานกองทุนสนับสนุนการวิจัย

สารบัญ

บทคัดย่อ 1

ปัจจัยเสี่ยงของภาวะตัวเหลืองในทารกแรกเกิดในภาคเหนือของประเทศไทย

วัตถุประสงค์ เพื่อประเมินอุบัติการณ์และปัจจัยเสี่ยงของภาวะตัวเหลืองในทารกแรกเกิดในภาคเหนือของประเทศไทย

วัสดุและวิธีการ ได้ศึกษาทารกแรกเกิดคลอดครบกำหนดที่โรงพยาบาลมหาราชนครเชียงใหม่แบบติดตามไปข้างหน้า ได้เก็บเลือดจากสายรกหลังจากที่ทารกคลอดแล้วและนำไปตรวจหมู่เลือดเอบีโอ ระดับเอนไซม์จี-ซิก-พีดี และภาวะอัลฟ่า-ธาลัสซีเมีย ตัวอย่างเลือดที่มีภาวะพร่องเอนไซม์จี-ซิก-พีดีจะนำไปตรวจหาความผิดปกติของยีนยูจีทีวันเอวัน (*UGT1A1* gene) ที่ตำแหน่งนิวคลีโอไทด์ 211 (G211A) และที่ตำแหน่งโปรโมเตอร์ (TA7) ใช้การตรวจทางสถิติแบบ univariate และ multivariate logistic regression เพื่อวิเคราะห์หาปัจจัยเสี่ยงของภาวะตัวเหลืองในทารกแรกเกิด

ผลการศึกษา ทารกแรกเกิดจำนวน 543 รายเข้าร่วมในการศึกษา ทารก 87 ราย (ร้อยละ 16) มีภาวะตัวเลืองที่ได้รับการรักษาด้วยการส่องไฟ ไม่มีทารกรายใดได้รับการถ่ายเลือด วิธีการคลอดด้วยเครื่องดูดสุญญากาศ ภาวะหมู่เลือดเอบีโอไม่เข้ากันระหว่างทารกและมารดา และภาวะพร่องเอนไซม์จี-ซิก-พีดี ทำให้ความเสี่ยงต่อการเกิดภาวะตัวเหลืองในทารกแรกเกิดเพิ่มขึ้น 2-4 เท่า ชนิดของนม ร้อยละของน้ำหนักตัวที่ลดลง ภาวะเลือดออกชนิด cephalohematoma การเป็นพาหะของอัลฟ่า-ธาลัสซีเมีย และโรคฮีโมโกลบิน เอช ไม่เป็นปัจจัยเสี่ยงของภาวะตัวเหลือง ความผิดปกติของยีนยูจีทีวันเอวันไม่เพิ่มความเสี่ยงของภาวะตัวเหลืองในกลุ่มทารกที่มีภาวะพร่องเอนไซม์จี-ซิก-พีดี

สรุป พบอุบัติการณ์ของภาวะตัวเหลืองในทารกแรกเกิดสูงในทารกแรกเกิดในภาคเหนือของประเทศไทย ภาวะหมู่เลือดเอบีโอไม่เข้ากันระหว่างทารกและมารดา และภาวะพร่องเอนไซม์จี-ซิก-พีดีซึ่งพบได้บ่อยในกลุ่มประชากร และวิธีการคลอดด้วยเครื่องดูดสุญญากาศ เป็นปัจจัยเสี่ยงที่สำคัญ

คำสำคัญ ภาวะตัวเหลืองในทารกแรกเกิด ประเทศไทย ภาวะพร่องเอนไซม์จี-ซิก-พีดี ภาวะหมู่เลือดเอบีโอไม่เข้ากันระหว่างทารกและมารดา อัลฟ่า-ธาลัสซีเมีย ความผิดปกติของยีนยูจีทีวันเอวัน

Abstract 1

Risk factors for hyperbilirubinemia in northern Thai newborns

Objective To determine the incidence and risk factors for neonatal hyperbilirubinemia in northern Thai newborns.

Study design A prospective cohort study of healthy full-term newborns was conducted at Chiang Mai University Hospital. Umbilical cord blood was collected after delivery and tested for ABO blood group, G6PD enzyme level, and α-thalassemia. The samples which were G6PD deficient were further tested for *UGT1A1* variants at nucleotide 211 (G211A) and TATA promoter (TA7). Univariate and multivariate logistic regression analyses were performed for risk factors for neonatal hyperbilirubinemia.

Results Five hundred and forty-three newborns were included into the study. Eighty-seven (16%) newborns had hyperbilirubinemia requiring phototherapy. None required exchange transfusion. Delivery by vacuum extraction, ABO blood group incompatibility and G6PD deficiency were significantly associated with twofold to fourfold increase in odds of neonatal hyperbilirubinemia. Type of feeding, percentage of weight loss, cephalohematoma, α^0-thalassemia carrier and hemoglobin H disease were not associated with neonatal hyperbilirubinemia. *UGT1A1* polymorphisms did not increase the incidence of hyperbilirubinemia in G6PD deficient newborns.

Conclusions The incidence of neonatal hyperbilirubinemia is high in northern Thai newborns. ABO blood group incompatibility and G6PD deficiency which are prevalent in the population, and also delivery by vacuum extraction are confirmed as strong risk factors.

Keywords: neonatal hyperbilirubinemia, Thailand, G6PD deficiency, ABO blood group incompatibility, α-thalassemia, *UGT1A1* polymorphism

บทคัดย่อ 2

การตรวจคัดกรองภาวะอัลฟ่า-ธาลัสซีเมียและฮีโมโกลบินผิดปกติโดยวิธีไอโซอิเลคทริคโฟกัสซิ่งในทารกแรกเกิดไทยในภาคเหนือ: ความสัมพันธ์ระหว่างจีโนไทป์และค่าทางโลหิตวิทยา

ได้รายงานการตรวจคัดกรองภาวะธาลัสซีเมียและฮีโมโกลบินผิดปกติโดยวิธีไอโซอิเลคทริคโฟกัสซิ่ง (Isoelectric focusing, IEF) ในทารกแรกเกิดไทยในภาคเหนือซึ่งเป็นพื้นที่ที่พบภาวะฮีโมโกลบินผิดปกติได้มาก การศึกษานี้เน้นผลการตรวจพบในภาวะอัลฟ่า-ธาลัสซีเมีย ฮีโมโกลบิน อี และฮีโมโกลบินผิดปกติชนิดอื่นๆ และรายงานผลการตรวจพบทางโลหิตวิทยาในแต่ละจีโนไทป์ (genotype) ทารกแรกเกิดจำนวน 207 ใน 566 ราย (ร้อยละ 36.6) มียีนธาลัสซีเมียหรือฮีโมโกลบินผิดปกติ พบจีโนไทป์ทั้งสิ้น 17 จีโนไทป์ ทารก 9 ราย (ร้อยละ 1.6) เป็นโรคฮีโมโกลบินเอช (5 ราย เป็นโรคฮีโมโกลบิน เอช ที่เกิดจากการขาดหายของยีน 2 รายเป็นโรคฮีโมโกลบินเอช/ฮีโมโกลบินคอนแสตนท์ สปริง 1 รายเป็นโรคฮีโมโกลบิน เอช ที่เกิดจากการขาดหายของยีนร่วมกับพาหะของฮีโมโกลบิน อี และ 1 รายเป็นโรคฮีโมโกลบินเอช/ฮีโมโกลบินคอนแสตนท์ สปริงร่วมกับพาหะของฮีโมโกลบิน อี) และพบทารก 1 รายเป็นโรคฮีโมโกลบินอี/เบต้า-ธาลัสซีเมีย วิธีไอโซอิเลคทริคโฟกัสซิ่งสามารถแยกโรคฮีโมโกลบินเอช และพาหะของอัลฟ่า-ธาลัสซีเมียชนิดที่มีความผิดปกติของสองยีน ออกจากทารกปกติได้ชัดเจนโดยดูจากปริมาณของฮีโมโกลบินบาร์ทที่ตรวจพบ สำหรับพาหะของอัลฟ่า-ธาลัสซีเมียชนิดที่มีความผิดปกติของยีนเดียวนั้น อาจตรวจไม่พบฮีโมโกลบินบาร์ทหรือพบในปริมาณน้อยทำให้ไม่สามารถใช้ในการวินิจฉัย ถ้าพบฮีโมโกลบินเพิ่มเติมจากปกติในตำแหน่งของฮีโมโกลบินเอทูจะบ่งชี้ว่าทารกเป็นพาหะของฮีโมโกลบิน อี ทารกรายหนึ่งที่ไม่มีฮีโมโกลบินเอและพบฮีโมโกลบิน อี ได้รับการวินิจฉัยเป็นโรคฮีโมโกลบินอี/เบต้า-ธาลัสซีเมีย พาหะของฮีโมโกลบินคิว-ไทยแลนด์ (Q-Thailand) สองรายมีฮีโมโกลบินผิดปกติเพิ่มเติมอีกสองชนิด ซึ่งน่าจะเป็นฮีโมโกลบินที่ประกอบด้วยแกมม่า-โกลบิน และเบต้า-โกลบินร่วมกับอัลฟ่า-โกลบินที่ผิดปกติ ทารกที่เป็นโรคฮีโมโกลบิน เอช มีระดับฮีโมโกลบิน ค่าขนาดโดยเฉลี่ยของเม็ดเลือดงแดง และค่าปริมาณของฮีโมโกลบินในเม็ดเลือดแดงน้อยกว่าปกติ ค่าขนาดโดยเฉลี่ยของเม็ดเลือดงแดงและค่าปริมาณของฮีโมโกลบินในเม็ดเลือดแดง สามารถใช้แยกพาหะของอัลฟ่า-ธาลัสซีเมียชนิดที่มีความผิดปกติของสองยีนได้ แต่ไม่สามารถใช้แยกพาหะของฮีโมโกลบิน อี หรือพาหะของอัลฟ่า-ธาลัสซีเมียชนิดที่มีความผิดปกติของยีนเดียว วิธีไอโซอิเลคทริคโฟกัสซิ่งเป็นวิธีการตรวจคัดกรองภาวะอัลฟ่า-ธาลัสซีเมียและฮีโมโกลบินผิดปกติในทารกแรกเกิดที่เชื่อถือได้

คำสำคัญ การตรวจคัดกรองในทารกแรกเกิด ธาลัสซีเมีย ฮีโมโกลบินผิดปกติ ไอโซอิเลคทริคโฟกัสซิ่ง

Abstract 2

Cord blood screening for α-thalassemia and hemoglobin variants by isoelectric focusing in northern Thai neonates: correlation with genotypes and hematologic parameters

We describe the screening of newborns for thalassemia and Hb variants by using isoelectric focusing (IEF) in a population from northern Thailand where hemoglobinopathies are highly prevalent. The report focuses on findings of α-thalassemia, Hb E, and other hemoglobin variants, and their correlation with genotypes and hematologic parameters. Two-hundred and seven out of 566 newborns (36.6%) had thalassemia genes or Hb variants. Seventeen different genotypes were found. Nine cases (1.6%) of Hb H disease (5 deletional Hb H diseases, 2 Hb H/Constant Spring diseases, 1 deletional Hb H disease/Hb E, carrier and 1 Hb H/Constant Spring disease/Hb E carrier) and one Hb E-β-thalassemia were identified. IEF could clearly distinguish Hb H diseases and carriers of two α-globin gene defects from normal individuals according to the presence of Hb Bart's and its percentage. For carriers of a single α-globin gene defect, Hb Bart's was either absent or present in a small amount and was therefore not reliable for screening. The presence of an additional band at the Hb A_2 position in the newborns signified an Hb E carrier. One case of an absent Hb A and a presence of Hb E was identified as Hb E-β-thalassemia. Two Hb Q-Thailand carriers were seen with two additional Hb fractions, presumably combinations of γ-globin and β-globin with the α-globin variant. Newborns with Hb H disease had lower Hb, MCV, and MCH levels than normal. MCV and MCH were also useful for differentiation of carriers of two α-globin gene defects, but not for carriers of Hb E or single α-globin gene defect. IEF was a reliable method for neonatal cord blood screening for α-thalassemia and Hb variants.

Keywords: newborn screening, thalassemia, hemoglobinopathies, isoelectric focusing

บทคัดย่อ 3

ความชุกและความผิดปกติในระดับยีนของภาวะพร่องเอนไซม์จี-ซิก-พีดี ในภาคเหนือของประเทศไทย

ได้ตรวจคัดกรองภาวะพร่องเอนไซม์จี-ซิก-พีดี โดยใช้เลือดสายรกจากทารกแรกเกิดในภาคเหนือของประเทศไทยจำนวน 566 ราย พบว่าทารก 90 ราย (ร้อยละ 16) มีภาวะพร่องเอนไซม์จี-ซิก-พีดี ความชุกของภาวะพร่องเอนไซม์จี-ซิก-พีดีในทารกเพศชายเท่ากับร้อยละ 17 (48 รายจากทารกเพศชาย 289 ราย) ความชุกของภาวะพร่องเอนไซม์จี-ซิก-พีดีบางส่วนในทารกเพศหญิงเท่ากับร้อยละ 13 (48 รายจากทารกเพศหญิง 277 ราย) และความชุกของภาวะพร่องเอนไซม์จี-ซิก-พีดีสมบูรณ์เท่ากับร้อยละ 1.8 (5 รายจากทารกเพศหญิง 277 ราย) ได้ตรวจหาความผิดปกติในระดับยีนที่ทำให้เกิดภาวะพร่องเอนไซม์จี-ซิก-พีดีที่เคยมีรายงานในประเทศไทย 6 ชนิด ได้แก่ จี-ซิก-พีดี เวียงจันทน์ (G6PD Viangchan, 871G>A) จี-ซิก-พีดี มหิดล (G6PD Mahidol, 487G>A) จี-ซิก-พีดี ไคปิง (G6PD Kaiping, 1388G>A) จี-ซิก-พีดี แคนตัน (G6PD Canton, 1376G>T) จี-ซิก-พีดี ยูเนี่ยน (G6PD Union, 1360C>T) จี-ซิก-พีดี ไชนีส-ไฟว์ (G6PD Chinese-5, 1024C>T) ด้วยวิธี polymerase chain reaction-restriction fragment length analysis จากอัลลีล 95 อัลลีลที่ตรวจสอบ พบว่า จี-ซิก-พีดี มหิดล (19) จี-ซิก-พีดี ไคปิง (17) จี-ซิก-พีดี แคนตัน (15) และจี-ซิก-พีดี เวียงจันทน์ (13) เป็นความผิดปกติที่พบบ่อยที่สุด ความผิดปกติในระดับยีนทั้งสี่ชนิดนี้รวมกันพบได้ประมาณร้อยละ 70 ของความผิดปกติในระดับยีนที่ทำให้เกิดภาวะพร่องเอนไซม์จี-ซิก-พีดีในประชากร สัดส่วนของจี-ซิก-พีดี มหิดล จี-ซิก-พีดี ไคปิง และจี-ซิก-พีดี แคนตันที่มากกว่าจี-ซิก-พีดี เวียงจันทน์ ซึ่งต่างจากที่พบในภาคอื่นของประเทศไทย แสดงให้เห็นถึงความสำคัญของการถ่ายโอนพันธุกรรมจากประเทศจีนตอนใต้และเมียนมาร์มายังพื้นที่นี้ และแสดงถึงความต่อเนื่องของลักษณะทางพันธุกรรมจากประเทศจีนตอนใต้และเมียนมาร์ มายังภาคเหนือและภาคกลางของประเทศไทย

คำสำคัญ ภาวะพร่องเอนไซม์จี-ซิก-พีดี ความผิดปกติในระดับยีน ภาคเหนือ ประเทศไทย คนไทย

Abstract 3

Prevalence and molecular characterization of glucose-6-phosphate dehydrogenase deficiency in northern Thailand

Neonatal cord blood screening for G6PD deficiency was conducted in 566 northern Thai newborns. Ninety (16%) had G6PD deficiency. The prevalence in male newborns was 17% (48 of 289 male newborns). The prevalence of female newborns having an intermediate deficiency and a complete deficiency of G6PD enzyme was 13% (37 of 277 female newborns) and 1.8% (5 of 277 female newborns) respectively. Six common G6PD variants previously reported in Thailand; G6PD Viangchan (871G>A), G6PD Mahidol (487G>A), G6PD Kaiping (1388G>A), G6PD Canton (1376G>T), G6PD Union (1360C>T) and G6PD Chinese-5 (1024C>T) were tested using polymerase chain reaction-restriction fragment length analysis. From 95 *G6PD* alleles tested, G6PD Mahidol (19), G6PD Kaiping (17), G6PD Canton (15) and G6PD Viangchan (13) are the most common mutations. These four mutations combined comprise about 70% of G6PD mutations in the population. The higher proportion of G6PD Mahidol, G6PD Kaiping, G6PD Canton in our population as opposed to G6PD Viangchan being the commonest mutations in the other parts of Thailand suggests a significant genetic drift from southern China and Myanmar into the region, and represents a genetic continuum from southern China and Myanmar to northern and central Thailand.

Keywords: Glucose-6-phosphate dehydrogenase/mutation/northern Thailand/Thais

Executive Summary

Neonatal hyperbilirubinemia in northern Thailand: roles of G6PD deficiency and alpha-thalassemia

Background

Neonatal hyperbilirubinemia is a common problem in Thai newborns. The most serious complication of neonatal hyperbilirubinemia is bilirubin encephalopathy which is caused by the direct toxic effects of deposited bilirubin to basal ganglia, pons and cerebellum. Affected newborns may present with seizures, developmental delay, or choreoathetiod cerebral palsy. Bilirubin encephalopathy is prevented by early detection of hyperbilirubinemia and early treatment with phototherapy and/or exchange blood transfusion and other supportive cares.

Causes of neonatal hyperbilirubinemia may be classified into those causing increased destruction of red blood cells and therefore increased bilirubin production and those causing impaired bilirubin conjugation or excretion.

After senescent red blood cells are destroyed mainly by splenic macrophages, hemoglobin is released and heme is converted to unconjugated bilirubin. Unconjugated bilirubin is bound by albumin and transported to liver, where bilirubin conjugation takes place. Conjugated bilirubin is excreted in bile. Mostly neonatal hyperbilirubinemia is a physiologic process which results from increased destruction of red blood cells as newborns have higher red blood cell mass than adults and the cells have shorter life-span (70-90 days). Also, newborns have lower level of albumin, immature conjugation function of liver and increased reuptake of bilirubin via enterohepatic circulation. All of which leads to increased level of unconjugated bilirubin.

Pathologic neonatal hyperbilirubinemia in term newborns is defined as visible jaundice or bilirubin level higher than 5 g/dL within the first 24 hours of life, increasing bilirubin level more than 0.2 mg/dL/hour or 5 mg/dL/day, bilirubin level higher than 95th percentile of age, prolonged hyperbilirubinemia longer than 2 weeks of age, or conjugated bilirubin level higher than 1.5-2 mg/dL.

The incidence of neonatal hyperbilirubinemia varies among races. Asian newborns are the most at risk, followed by the Caucasians and Blacks, respectively. Genetic characterisitcs, including higher prevalence of G6PD deficiency and ABO blood group

incompatibility and possibly thalassemia, may contribute to the higher incidence of neonatal hyperbilirubinemia in Asians.

The primary objective of this project is to determine the incidence and risk factors for neonatal hyperbilirubinemia in Thai newborns. The studied risk factors include clinical and genetic factors common in the population.

The secondary objectives of this project are to demonstrate the hematological and hemoglobin parameters of newborns with thalassemia and hemoglobin variants, and to determine the prevalence of G6PD deficiency and molecular characterization of G6PD variants.

There are three studies within this project:

In the first study, we studied the incidence, risk factors and characteristics of neonatal hyperbilirubinemia in northern Thailand, focusing on roles of G6PD deficiency and alpha-thalassemia, which are common causes of hemolytic anemia in northern Thailand, on the development of neonatal hyperbilirubinemia. We also studied the role of uridine diphosphoglucuronosyl transferase 1A1 (*UGT1A1*) polymorphisms on their role of increasing the incidence of neonatal hyperbilirubinemia in G6PD deficient newborns. The *UGT1A1* gene encodes UGT enzyme which functions in bilirubin conjugation.

In the second study, we looked at hematological parameters and hemoglobin patterns of newborns with thalassemia and hemoglobin variants.

In the third study, we studied the prevalence of G6PD deficiency and molecular characterization of G6PD variants.

Materials and methods

This is a prospective cohort study. The study protocol was approved by the Institutional Ethics Committee. Informed consent was obtained from the mothers. Full-term newborns (gestational age 37-42 weeks) of mothers with uneventful pregnancy who were born at Chiang Mai University Hospital were included. Newborns with known maternal history of Rh(D) incompatibility, diabetes mellitus, and newborns with congenital anomalies, infection requiring antibiotics or significant hemorrhages other than cephalohematoma were excluded from clinical analysis.

The umbilical cord blood was collected after delivery of the newborn. The blood samples were kept at $4^{\circ}C$ until analysis. The samples were tested for complete blood count, ABO blood group, G6PD enzyme level, and α-thalassemia by isoelectric focusing (IEF)

method and mutation-specific polymerase chain reactions. The samples which were G6PD deficient were further tested for *UGT1A1* polymorphisms at nt 211 and TATA promoter region, and mutation-specific polymerase chain reactions for G6PD mutations.

Clinical features were summarized for newborns with and without hyperbilirubinemia. Univariate and multivariate binary logistic analyses were performed to determine the risk factors for neonatal hyperbilirubinemia.

Hematologic and hemoglobin parameters were summarized for each α-globin and β^E genotypes.

Prevalence of G6PD deficiency was determined and molecular characteristics of G6PD mutations were summarized.

Results

Five hundred and sixty-six newborns were enrolled into the study, and 543 were included for clinical analysis. Eighty-seven (16%) newborns had hyperbilirubinemia requiring phototherapy. None required exchange transfusion. Delivery by vacuum extraction, ABO blood group incompatibility and G6PD deficiency were significantly associated with twofold to fourfold increase in odds of neonatal hyperbilirubinemia. Type of feeding, percentage of weight loss, cephalohematoma, α^0-thalassemia carrier and hemoglobin H disease were not associated with neonatal hyperbilirubinemia. *UGT1A1* polymorphisms did not increase the incidence of hyperbilirubinemia in G6PD deficient newborns.

Two-hundred and seven out of 566 newborns (36.6%) had thalassemia genes or Hb variants. Seventeen different genotypes were found. Nine cases (1.6%) of Hb H disease (5 deletional Hb H diseases, 2 Hb H/Constant Spring diseases, 1 deletional Hb H disease/Hb E, carrier and 1 Hb H/Constant Spring disease/Hb E carrier) and one Hb E-β-thalassemia were identified. IEF could clearly distinguish Hb H diseases and carriers of two α-globin gene defects from normal individuals according to the presence of Hb Bart's and its percentage. For carriers of a single α-globin gene defect, Hb Bart's was either absent or present in a small amount and was therefore not reliable for screening. The presence of an additional band at the Hb A_2 position in the newborns signified an Hb E carrier. One case of an absent Hb A and a presence of Hb E was identified as Hb E-β-thalassemia. Two Hb Q-Thailand carriers were seen with two additional Hb fractions, presumably combinations of γ-globin and β-globin with the α-globin variant. Newborns with Hb H disease had lower Hb, MCV, and MCH levels than normal. MCV and MCH were also useful for differentiation of

carriers of two α-globin gene defects, but not for carriers of Hb E or single α-globin gene defect.

Ninety (16%) of 566 newborns had G6PD deficiency. The prevalence in male newborns was 17% (48 of 289 male newborns). The prevalence of female newborns having an intermediate deficiency and a complete deficiency of G6PD enzyme was 13% (37 of 277 female newborns) and 1.8% (5 of 277 female newborns) respectively. From 95 *G6PD* alleles tested for common G6PD variants, G6PD Mahidol (20), G6PD Kaiping (18), G6PD Canton (16) and G6PD Viangchan (14) are the most common mutations. These four mutations combined comprise about 70% of G6PD mutations in the population.

Conclusions

The incidence of neonatal hyperbilirubinemia is high in northern Thai newborns. ABO blood group incompatibility and G6PD deficiency which are prevalent in the population, and also delivery by vacuum extraction are confirmed as strong risk factors.

IEF was a reliable method for neonatal cord blood screening for α-thalassemia and Hb variants.

The prevalence of G6PD deficiency is high, affecting 17% of male newborns. The higher proportion of G6PD Mahidol, G6PD Kaiping, G6PD Canton in our population as opposed to G6PD Viangchan being the commonest mutations in the other parts of Thailand suggests a significant genetic drift from China and Myanmar into the region.

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Study 1

Risk factors for hyperbilirubinemia in northern Thai newborns

Introduction

Early identification and management of severe neonatal hyperbilirubinemia is important to prevent its most devastating complication, bilirubin encephalopathy. Clinical and genetic factors each play a role in neonatal hyperbilirubinemia.[1] The major risk factors as stated in clinical practice guidelines on hyperbilirubinemia management by American Academy of Pediatrics (AAP) in 2004 include late-preterm gestational age, exclusive breast feeding, blood group incompatibility, hemolytic diseases such as glucose-6-phosphate dehydrogenase (G6PD) deficiency, East Asian race, cephalohematoma or significant bruising and history of previous sibling received phototherapy.[2] Several reported genetic risk factors are polymorphisms of genes modulating bilirubin metabolism, such as the uridine diphosphoglucuronosyl transferase 1A1 (*UGT1A1*) gene that encodes UGT enzyme which involves bilirubin conjugation, and solute carrier organic anion transporter family member 1B1 *(SLCO1B1)* gene that encodes transporting polypeptide which functions in hepatic bilirubin uptake.[1,3-5]

The incidence of non-physiologic hyperbilirubinemia in Asian newborns has been reported to be as high as more than 30%.[6] ABO blood group incompatibilities and G6PD deficiency plus environmental exposure to offending agents are common in the population and may partly explain the high incidence. *UGT1A1* variant at nucleotide (nt) 211 (G211A) and *SLCO1B1* at nt 388 (A388G) are two major variants reported to associate with neonatal hyperbilirubinemia in east Asian newborns.[7-10] *UGT1A1* TATA promoter variant (TA7) which is the risk factor in Caucasians is less common in the population.[8,9]

UGT1A1 polymorphisms are particularly seen in prolonged neonatal hyperbilirubinemia, and especially in exclusively breast-fed newborns.[4,7-9] Also, they are found to be an additive risk factor for neonatal hyperbilirubinemia in G6PD deficient newborns.[5,11,12]

Thailand is a country in Southeast Asia. Reported risk factors of hyperbilirubinemia in Thai newborns are similar to those in East Asian newborns.[13,14] G6PD deficiency and ABO incompatibility which are prevalent in Thais are the main risk factors. Additionally, delivery by vacuum extraction is reportedly a risk factor.[14] *UGT1A1* variant at nt 211, but not *UGT1A1* variant at nt 686 (C686A) or polymorphisms of *SCLO1B1* and *GST* (glutathione S-transferase) genes, were recently reported to be a risk factor for hyperbilirubinemia in Thai newborns.[15]

Hemolytic anemias cause increased bilirubin production, and are therefore a significant cause of neonatal hyperbilirubinemia. In northern Thailand, hemoglobin H (Hb H) disease is a highly prevalent hereditary hemolytic anemia.[16] The condition is caused by deletions with or without point mutations of three out of four normally functioning α-globin genes, leaving one intact gene (— — /— α or — — /α^Tα).[17] The excess β-globin combines to form Hb H which is unstable and has a high affinity for oxygen and is therefore ineffective for oxygen transportation. Patients with Hb H disease generally have mild to moderate anemia. They may present with acute hemolysis triggered by fever or infections. Some reportedly present early in life with neonatal hyperbilirubinemia.[18,19]

There are two types of α-thalassemia carriers, α^0-thalassemia and α^+-thalassemia. The former results from *in-cis* deletion of two α-globin genes and the latter results from deletion or mutation of a single α-globin gene. α^0-thalassemia carriers have hypochromic and microcytic red blood cells. During the newborn period, they have elevated Hb Bart's which demonstrates an imbalance production of alpha- and gamma-globins.[20] They are also shown to have lower Hb level *in utero* from mid-gestation and during the newborn period; this finding demonstrates an important effect of a decreased α-globin production.[20,21] Any additional role of hemolysis on the lower Hb levels in the fetal and newborn periods is unknown. α^+-thalassemia carriers have minimally raised Hb Bart's at birth and are typically asymptomatic.[20]

While Hb H disease is conceivably a risk for neonatal hyperbilirubinemia, the evidence is limited. α^0-thalassemia carrier status was shown to pose no additional risk to newborns with G6PD deficiency.[22] On the contrary, it was shown to be protective in Taiwanese newborns.[23]

The objectives of this study were to determine the incidence of neonatal hyperbilirubinemia and its associated clinical and genetic factors in a cohort study of full-term healthy newborns from northern Thailand. The roles of mode of delivery, type of feeding, degree of weight loss, G6PD deficiency, ABO blood group incompatibilities, α^0-thalassemia carriers and Hb H disease on hyperbilirubinemia were investigated. *UGT1A1* gene polymorphisms were further explored in cases with G6PD deficiencies for their additive roles on neonatal hyperbilirubinemia.

Materials and Methods

This is a prospective cohort study. The study protocol was approved by the Institutional Ethics Committee. Informed consent was obtained from the mothers. Full-term newborns (gestational age 37-42 weeks) of mothers with uneventful pregnancy who were born at Chiang Mai University Hospital were included. Newborns with known maternal history of Rh(D) incompatibility, diabetes mellitus, and newborns with congenital anomalies, infection requiring antibiotics or significant hemorrhages other than cephalohematoma were excluded from the study.

The umbilical cord blood was collected after delivery of the newborn. The blood samples were kept at $4^{\circ}C$ until analysis. The samples were tested for ABO blood group, G6PD enzyme level, and α-thalassemia. The samples which were G6PD deficient were further tested for *UGT1A1* polymorphisms at nt 211 and TATA promoter region.

ABO blood group was determined by a standard technique. ABO incompatibility was defined as maternal blood group O and newborn A or B. G6PD assay was performed according to the WHO method within 7 days of blood collection.[24] Average G6PD level in cord blood from male newborns was 12.5±2.3 IU/g Hb. Complete and intermediate deficiency of G6PD enzyme were defined by a level of less than 1.5 and 1.5-8.0 IU/g Hb respectively. Carriers of Southeast-Asian deletional α^{0}-thalassemia, the most common α^{0}-thalassemia in the area, and Hb H disease were diagnosed by polymerase chain reaction and Hb analysis as described previously.[20]

UGT1A1 polymorphisms at nt 211 and TATA promoter region were identified by methods as described previously.[8,25]

Records of newborn admissions were reviewed for clinical features including: gender, gestational age, birth weight and percentage of weight loss from birth within the admission, Apgar score at 5 minutes, delivery method, occurrence and identified causes of neonatal hyperbilirubinemia requiring phototherapy or exchange transfusion. The decision for treatment of neonatal hyperbilirubinemia was up to the pediatric residents and attending neonatologists, who were encouraged to follow the AAP 2004 guideline.[2] Lower threshold of bilirubin level for phototherapy may be used for newborns with undefined risk factors who awaited laboratory results. At our hospital, healthy newborns are usually discharged after blood sampling for thyroid and phenylketonuria screening after 48 hours. Newborns with suspected hyperbilirubinemia are kept in the hospital longer for observation and treatment as needed.

Clinical features were summarized for newborns with and without hyperbilirubinemia. Univariate binary logistic analysis was performed for delivery method, presence of cephalohematoma, type of feeding, percentage of weight loss, G6PD enzyme status, ABO blood group incompatibilities, and α-thalassemia status. Factors that were found to be significantly associated with neonatal hyperbilirubinemia were further evaluated by multivariate binary logistic analysis for adjusted odds ratio and their 95% confidence intervals (CI). Incidence of neonatal hyperbilirubinemia in G6PD deficient newborns was compared between groups with and without *UGT1A1* polymorphisms using Chi-square test. P-values of less than 0.05 were considered statistically significant.

Results

Five hundred and sixty-six newborns were enrolled in the study. Twenty-three were excluded for the following reasons: 9 congenital anomalies, 8 infections requiring treatment with antibiotics, 2 maternal class A1 diabetes mellitus, 2 known significant hemorrhage (1 intracranial hemorrhage and 1 large hematoma), and 1 each of Rh (D) incompatibility and maternal hepatitis B virus carrier.

The clinical features of the included 543 newborns are shown in Table 1. There were 278 (51.2%) male newborns. Mean gestational age was 38.7±1.1 weeks. All had Apgar scores at 5 minutes of not less than 8. Eighty-seven (16%) newborns had hyperbilirubinemia requiring phototherapy. None required exchange transfusion. Causes of neonatal hyperbilirubinemia were identified in 39 (44.8%) newborns: 26 G6PD deficiency, 7 Coombs' positive ABO blood group incompatibility, 3 concurrent G6PD deficiency and ABO blood group incompatibility, 2 cephalohematoma, and 1 Hb H disease. A newborn with deletional Hb H disease who had hyperbilirubinemia had no other known risk factors; he was the first child of the family, born by normal delivery, had a birth weight of 2,700 g, had elevated G6PD level (31.05 u/g Hb). Both mother and child had A, Rh(D) positive blood group. The result of direct and indirect Coombs' tests on the child's blood were negative. All 9 newborns with Hb H disease (5 deletional Hb H disease, 2 Hb H/Hb Constant Spring (CS) disease, 1 AEBart's disease and 1 AEBart'sCS disease) had elevated G6PD levels (24.4±3.0 u/g Hb, range 21.1-31.1 u/g Hb).

Table 1: Clinical features of newborns classified by the presence or absence of neonatal hyperbilirubinemia (NH)

Clinical features	**NH**	**Non NH**	**p-value**
Number, n (%)	87 (16.0)	456 (84.0)	-
Male gender, n (%)	45 (51.7)	233 (51.1)	0.92
Gestational age, mean±SD, week	38.5±1.1	38.8±1.1	0.10
Birth weight, mean±SD, g	3,026±380	3,138±349	0.007
Birth weight < 2,500 g, n (%)	5 (5.7)	9 (2.0)	0.05
Percentage of weight loss from birth within the admission, mean±SD, %	5.8±2.4	5.5±2.3	0.37

Tables 2 and 3 respectively show the odds ratio and 95% CI for factors obtained by univariate and multivariate binary logistic analyses. Delivery by vacuum extraction, ABO blood group incompatibility and G6PD deficiency were associated with development of neonatal hyperbilirubinemia. Cephalohematoma, type of feeding, percentage of weight loss, α^0-thalassemia carrier status and Hb H disease were not associated with neonatal hyperbilirubinemia.

Table 2: Univariate analysis of risk factors for neonatal hyperbilirubinemia

Factors	**NH** **n (%)**	**Non NH** **n (%)**	**Odds ratio** **(95% CI)**	**p-value**
Delivery method				
-Normal delivery	72 (82.8)	396 (86.8)	reference group	
-Vacuum extraction	11 (12.6)	18 (3.9)	3.4 (1.5-7.4)	0.003
-Forceps extraction	2 (2.3)	3 (0.7)	3.7 (0.6-22.3)	0.16
-Breech extraction	1 (1.1)	2 (0.4)	2.8 (0.2-30.7)	0.41
-Caesarian section	1 (1.1)	37 (8.1)	0.15 (0.02-1.10)	0.06
Cephalohematoma				
-Presence	2 (2.3)	4 (0.9)	2.7 (0.5-14.7)	0.25
-Absence	85 (97.7)	452 (99.1)	reference group	
Type of feeding				
-Exclusive breast feeding	25 (28.7)	146 (32.0)	0.9 (0.5-1.4)	0.55
-Breast and supplementary feeding	62 (71.3)	310 (68.0)	reference group	
ABO blood group				
- Mother O/infant A or B	19 (21.8)	53 (11.6)	2.1 (1.2-3.7)	0.02
- Other	66 (75.9)	382 (83.8)	reference group	
-Unknown	2 (2.3)	21 (4.6)		
G6PD enzyme status				
-Normal	58 (66.7)	398 (87.3)	reference group	
-Intermediate deficiency	11 (12.6)	26 (5.7)	2.9 (1.4-6.2)	0.006
-Complete deficiency	18 (20.7)	32 (7.0)	3.9 (2.0-7.3)	<0.001
Percentage of weight loss				
-> 10% weight loss	3 (3.4)	12 (2.6)	1.3 (0.4-4.7)	0.69
-$\leq$ 10% weight loss	83 (95.4)	432 (94.7)	reference group	
-Unknown	1 (1.1)	12 (2.6)		
Alpha-thalassemia status				
-Non α^0-thalassemia carrier	82 (94.3)	425 (93.2)	reference group	
-α^0-thalassemia carrier	4 (4.6)	23 (5.0)	0.9 (0.3-2.7)	0.90
-Hemoglobin H disease	1 (1.1)	8 (1.8)	0.7 (0.1-5.3)	0.65

Table 3: Multivariate analysis of risk factors for neonatal hyperbilirubinemia

Factors	**Adjusted odds ratio (95% CI)**	**p-value**
Delivery method		
-Normal delivery	reference group	
-Vacuum extraction	4.2 (1.9-9.7)	0.001
-Forceps extraction	4.1 (0.6-27.2)	0.14
-Breech extraction	2.6 (0.2-33.0)	0.45
-Caesarian section	0.15 (0.02-1.1)	0.06
ABO blood group		
- Mother O/infant A or B	2.1 (1.1-3.9)	0.02
- Other	reference group	
G6PD enzyme status		
-Normal	reference group	
-Intermediate deficiency	3.4 (1.5-7.6)	0.004
-Complete deficiency	4.2 (2.1-8.2)	<0.001

Allele frequencies of *UGT1A1* polymorphisms at nt 211 and TATA promoter region were 0.15 and 0.16 respectively. Neither was associated with neonatal hyperbilirubinemia in G6PD deficient newborns. The results are shown in Table 4.

Table 4: *UGT1A1* polymorphisms in G6PD deficient newborns classified by the presence or absence of neonatal hyperbilirubinemia

***UGT1A1* polymorphisms**	**NH**	**Non NH**	**Total**	**p-value**
Nucleotide 211 (G>A)				
-G/G	20 (71.4)	40 (71.4)	60 (71.4)	0.77
-G/A	8 (28.6)	15 (26.8)	23 (27.4)	
-A/A	0	1 (1.8)	1 (1.2)	
TATA promoter (TA7)				
-TA6/6	22 (75.9)	37 (66.1)	59 (69.4)	0.55
-TA6/7	7 (24.1)	18 (32.1)	25 (29.4)	
-TA7/7	0	1 (1.8)	1 (1.2)	

Discussion

This is a comprehensive study of the roles of clinical and genetic factors in the development of neonatal hyperbilirubinemia in northern Thai newborns. The study shows a high incidence of neonatal hyperbilirubinemia necessitating treatment in the population, and emphasizes the important roles of factors including G6PD deficiency, ABO blood group incompatibilities, and delivery by vacuum extraction, in neonatal hyperbilirubinemia. The study also demonstrates that cephalohematoma, type of feeding, percentage of weight loss, α^0-thalassemia carriers and Hb H disease are not associated with neonatal hyperbilirubinemia. *UGT1A1* polymorphisms common in the population are not an additive risk factor for neonatal hyperbilirubinemia in G6PD deficient newborns.

G6PD deficient newborns were at significantly higher risk for neonatal hyperbilirubinemia. Hemizygous male and homozygous female G6PD-deficient newborns were at higher risk for neonatal hyperbilirubinemia than their heterozygous counterparts with intermediate deficiency of G6PD enzyme (adjusted odds ratio 4.2 and 3.4, respectively). This demonstrated the dosage effect of G6PD deficiency on bilirubin metabolism. This finding agrees with previous studies, and indicates that female carriers with intermediate deficiency of G6PD enzyme are also at risk and should be monitored for neonatal hyperbilirubinemia.[26,27]

ABO blood group incompatibilities were an important factor for neonatal hyperbilirubinemia. In this study, Coombs' tests were not routinely done, so it was not possible to distinguish the effects of Coombs' positive and negative ABO blood group incompatibilities on the development of neonatal hyperbilirubinemia.

Delivery by vacuum extraction was a risk for neonatal hyperbilirubinemia. The finding agrees with previous reports.[14,28] The effect might be explained by an inevitably higher possibility of soft tissue trauma and hemorrhage associated with the procedure. Justifiably, Caesarian section seemed to be a protective factor, although the difference was not significant. Cephalohematoma was not a risk factor. However the number of events was small.

α^0-thalassemia carriers and Hb H disease were neither a risk nor a protective factor for neonatal hyperbilirubinemia. This suggested negligible or no hemolysis in α^0-thalassemia carriers. However, increased hemolysis was evident in Hb H disease as indirectly indicated by elevated G6PD levels in all 9 Hb H newborns. In spite of this, only 1 had neonatal hyperbilirubinemia. This might imply that only a small number of newborns with Hb H disease experienced hemolysis that was serious enough to lead to neonatal

hyperbilirubinemia. This interesting finding needs confirmation in larger Hb H disease population.

UGT1A1 polymorphisms at nt 211 and TATA promoter region were previously reported to associate with higher bilirubin levels and gallstones in Thai Hb E/β-thalassemia patients.[29] Prachukthum *et al* reported that only *UGT1A1* polymorphisms at nt 211 was related to neonatal hyperbilirubinemia in a case-control study of newborns from central Thailand.[15] In this study, the gene frequency of the *UGT1A1* polymorphism at nt 211 polymorphism was slightly higher and that the polymorphism at TATA promoter region was comparable with these previous studies. However, neither polymorphism increased the incidence of neonatal hyperbilirubinemia in G6PD deficient newborns. Our finding agrees with previous studies that *UGT1A1* polymorphisms are not a risk factor for hyperbilirubinemia in the G6PD deficient newborns.[30,31] *UGT1A1* polymorphisms were reported to be an important risk for prolonged hyperbilirubinemia, especially in exclusively breast-fed newborns.[4,7-9] A shorter period of observation and a lower portion of exclusively breast-fed newborns in this study might have masked the effect of the polymorphisms.

Type of feeding was not different between the group with and without neonatal hyperbilirubinemia. However, the exact amount of supplementary feeding in each newborn was difficult to determine. This may have confounded the result in the supplementary group. Percentage of weight loss was used as an indicator of dehydration. It was also found not associated with neonatal hyperbilirubinemia. This again may be confounded by treatment as supplementary feeding was usually given for newborns who lost more than a predetermined percentage of birth weight.

The strength of this study is the large study population and the high prevalence of G6PD deficiency, ABO incompatibility and α-thalassemia, which allow for an identification of the roles of each factor. The weakness of this study is that as we collected the clinical data from hospital records, late-onset hyperbilirubinemia and readmissions to other hospitals might have been missed. Another remark is that the rate of phototherapy may be higher as some newborns were given phototherapy at lower bilirubin threshold while awaiting investigation results.

This study confirms a high incidence of neonatal hyperbilirubinemia in northern Thai population. It substantiates the roles of clinical factors including G6PD enzyme deficiency, ABO blood group incompatibility, and delivery by vacuum extraction, and refutes the roles of type of feeding, weight loss, α-thalassemia and common *UGT1A1* polymorphisms in combination with G6PD deficiency on neonatal hyperbilirubinemia.

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Study 2

Cord blood screening for α-thalassemia and hemoglobin variants by isoelectric focusing in northern Thai neonates: correlation with genotypes and hematologic parameters

Introduction

Newborn screening for hemoglobinopathies aims to detect thalassemia syndromes and hemoglobin (Hb) variants with significant effects to health, which would allow prevention of complications, early management, and genetic counseling.[1,2] Northern Thailand is among areas in the world where both α- and β-hemoglobinopathies are highly prevalent and interactions between different thalassemia genes lead to a diversity of thalassemia genotypes.[3-7] Through prenatal screening and diagnosis, severe thalassemia diseases, including homozygous α-thalassemia 1 causing Hb Bart's hydrops fetalis, homozygous β-thalassemia, and Hb E-β-thalassemia are usually detected prenatally.[8] On the other hand, Hb H disease, a condition with mild to moderate anemia caused by defects of three α-globin genes leaving only one intact gene, is usually not searched for. Although generally of mild severity, patients with Hb H disease may develop acute hemolysis necessitating blood transfusion when they have fever. Some are transfusion dependent, and there have been reports of hydrops fetalis associated with non-deletional Hb H disease.[9,10] The prevalence of Hb H disease is as high as 1:65 in the northern Thai population.3 Early identification from the newborn period will facilitate proper parental education, and prevent complications, unnecessary investigation, or iron treatment. Identification of carriers of thalassemia or Hb variants, especially for Hb E which is common in the population, will also be useful for family genetic counseling.

Several Hb analytic methods have been used for this purpose, including starch gel electrophoresis, cellulose acetate gel electrophoresis, isoelectric focusing (IEF), and high pressure liquid column chromatography (HPLC).[11-20] Currently IEF and HPLC are widely used. These methods can detect α-thalassemia carriers and diseases, β-thalassemia diseases and most Hb variants. Identification of β-thalassemia carriers requires molecular study, although lower Hb A amount has been shown to correlate with β-thalassemia carriers and may be used as a primary screen.[11,13] Identification of α-thalassemia depends on the presence of Hb Bart's, which reflects an imbalance of α-globin and γ-globin synthesis.[15-21] The findings are seen most distinctively during the fetal and neonatal periods, when the γ-globin production is active. Hb variants are characterized by the presence of a varying amount of Hb with different electrophoretic properties than the normal pattern of Hb F and Hb A in newborns.

IEF is an electrophoretic separation of different proteins according to their isoelectric points (pIs). A pH gradient is created by applying electrical current to an agarose gel

containing low molecular weight amphoteric molecules with varying pIs. Each Hb focuses at the pH position which corresponds to its pI.[22] IEF of Hbs in normal newborns typically shows Hb F and Hb A. Acetylated Hb F (Hb Fac), which is a derivative of Hb F, is also usually seen at approximately 10% of Hb F.[21] Quantitation of each Hb fractions can be further performed by a densitometric analysis of the gel. IEF has the advantage of high discriminatory ability.[12,21,23] The method is also highly sensitive, which is desirable for newborn screening when some Hb variants are present in small amounts. However, the method requires technical skills and reference values of different thalassemia syndromes, and Hb variants prevalent in the target population need to be established.

Herein, we report on newborn screening for thalassemia syndromes and Hb variants using IEF in a northern Thailand population where hemoglobinopathies are highly prevalent. This report focuses on findings of α-thalassemia, Hb E, and other hemoglobin variants, and their correlation with genotypes and hematologic parameters.

Materials and Methods

The study protocol was approved by the institutional ethics committee. Informed consent was obtained from the mothers. Five-hundred and sixty-six full-term newborns (gestational age 37-42 weeks) of mothers with uneventful pregnancy who were born at Chiang Mai University Hospital were included. After delivery of the newborn, the umbilical cord was cleaned with povidone iodine, and an 18-gauge needle was inserted into an umbilical vein to collect 5 mL of blood samples in EDTA. The blood samples were kept at 4^{o}C until analysis.

The Hb level and red cell indices were determined with an electronic cell counter (Beckman Coulter A^{C}·T 5diff Hematology Analyzer) within 48 hours of blood collection. IEF was performed within 4 weeks of blood collection using a Resolve Hemoglobin Kit (PerkinElmer Life and Analytical Sciences, Turku, Finland) as per the manufacturer's recommendation. After electrophoresis, the gels were scanned and analyzed by IsoScan® software (PerkinElmer Life and Analytical Sciences, Turku, Finland). Firstly the Hb fractions were outlined and percentages were automatically determined by the software program. Then the results were re-examined by two staff members for faint bands that were not automatically detected. The faint bands were outlined manually.

Genomic DNA was extracted from leukocytes by Chelex method.[24] Alpha-globin gene mutations were identified by polymerase chain reaction for three α-globin gene deletions: Southeast-Asian (- $-^{SEA}$), 3.7 kb ($-\alpha^{-3.7}$) and 4.2 kb ($-\alpha^{-4.2}$) deletions; and a point mutation at termination codon causing Hb Constant Spring (Hb CS, $\alpha^{142\ T-C}\alpha$) by methods described previously.[25-27]

Amplification refractory mutation system (ARMS)-polymerase chain reaction (PCR) for Hb Q-Thailand ($-\alpha^{74\ G-C}$) mutation was performed in 2 samples to confirm the diagnosis of Hb Q-Thailand carrier. Three primers were used: Hb Q-S (5'-AGT ATG GTG CGG AGG CCC TGG AGA-3'), a common upstream primer; Hb Q-N (5'-CAG GGC GGA CAG CGC GTT GGG CAT GTC GTC-3'); and Hb Q-M1 (5'-CAG GGC GGA CAG CGC GTT GGG CAT GTC GAG-3'). The Hb Q-N and Hb Q-S produced a 293 bp fragment from normal templates and the Hb Q-M1 and Hb Q-S produced a 293 bp fragment from mutational templates. Two primers were used as controls: AE12 (5'-TGC AAT CAT TCG TCT GTT TCC C-3') and SE12 (5'-AGA AGA GCC AAG GAC AGG TAC G). The control product was 660 bp in length. The PCR mixture (25 μL) contained 8 μL DNA, 0.1 μmol/L of each primer, 200 μmol/L dNTPs, 1 unit of Platinum® Taq DNA polymerase (Qiagen, Germany),

in 1X PCR buffer (20 mmol/L tris-HCl (pH8.4) and 50 mmol/L KCl), and 1.5 mmol/L $MgCl_2$. After a 15-minute initial denaturation at 95°C, 35 cycles of 94°C 60 s, 56°C 60 s and 72°C 2 min were performed on a GeneAmp PCR system 9700 (Perkin Elmer, CT, USA). The PCR products were analyzed by electrophoresis on a 2% agarose gel containing 0.5 mg/mL of ethidium bromide. The DNA bands were detected by UV light and documented using a Bio-Rad Gel Doc 1000 system.

Identification of HbE (β26 G-A) mutation was performed by an ARMSPCR in 65 samples with the presence of an additional band at the Hb A2 position to confirm the diagnosis of Hb E carrier. The method was as described previously.[28] ARMS-PCR for β-thalassemia and real-time PCR for β-globin gene deletion (β3.5 kb del) were performed on a case with Hb E-β-thalassemia by methods described previously.[28,29]

Records of newborn admissions were reviewed. Factors that may interfere with hematologic parameters, such as infections, maternal diabetes mellitus, Rh(D) compatibility, or congenital anomalies, were searched for. If any of these factors were present, only the results of globin genotypes and IEF were included for analysis; hematologic parameters were excluded.

Hematologic and hemoglobin parameters were summarized for each α-globin and β^E genotypes and were expressed as means and standard deviations.

Results

Two hundred and seven out of 566 newborns (36.6%) had thalassemia genes or Hb variants. Alpha-thalassemia 2 (3.7 and 4.2 kb deletions) carrier was the most common genotype (15.0%), followed by Hb E carrier (7.2%), α-thalasemia 1 (Southeast Asian deletion) carrier (4.9%), Hb CS carrier (2.7%), and Hb Q-Thailand carrier (0.4%). Nine newborns (1.6%) had Hb H disease: 5 cases of deletional Hb H disease ($--^{SEA}/-\alpha^{3.7}$), 2 non-deletional Hb H disease ($--^{SEA}/\alpha^{CS}\alpha$), and 2 double heterozygous Hb H disease and Hb E carrier. One was a compound heterozygote for Hb E-β-thalassemia ($\beta^{3.5\ kb\ del}/\beta^{26\ G\text{-}A}$). Overall, there were 17 thalassemia and Hb variant genotypes. Their prevalence is shown in Table 1. The Hb patterns seen in each genotype are shown in Table 2.

Table 1 Genotypes of thalassemia and hemoglobin variants

Genotype		Number (%)
Single α-globin gene defect	αα/-$\alpha^{-3.7}$	80 (14.1)
	αα/-$\alpha^{-4.2}$	5 (0.9)
	αα/α^{CS}α	15 (2.7)
Two α-globin gene defect	-$\alpha^{-3.7}$/-$\alpha^{-3.7}$	4 (0.7)
	-$\alpha^{-3.7}$/ α^{CS}α	1 (0.2)
	αα/- -^{SEA}	28 (4.9)
Hb H disease	- -^{SEA}/-$\alpha^{-3.7}$	5 (0.9)
	- -^{SEA}/ α^{CS}α	2 (0.4)
α-globin variant	αα/-$\alpha^{Q\text{-Thailand}}$	2 (0.4)
Normal α-globin gene and Hb E carrier	αα/αα, β/β^{E}	41 (7.2)
Single α-globin gene defect and Hb E carrier	αα/-$\alpha^{-3.7}$, β/β^{E}	14 (2.4)
	αα/α^{CS}α, β/β^{E}	5 (0.9)
Two α-globin gene defect and Hb E carrier	αα/--^{SEA}, β/β^{E}	1 (0.2)
	-$\alpha^{-3.7}$/ α^{CS}α, β/β^{E}	1 (0.2)
Hb H disease and Hb E carrier	- -^{SEA}/-$\alpha^{-3.7}$, β/β^{E}	1 (0.2)
	--^{SEA}/ α^{CS}α, β/β^{E}	1 (0.2)
Hb E- β-thalassemia	αα/αα, β^{0}/β^{E}	1 (0.2)
Normal α-globin gene	αα/αα	359 (63.4)
Total		566 (100)

Table 2 Hemoglobin patterns in correlation with genotypes

Genotype	Number (562)	Hb Bart's (%)	Hb A (%)	Hb A_2/E (%)	Hb variant (%)
Normal α-globin gene (αα/αα)	357	0.02±0.12	20.7±6.2	-	-
Single α-globin gene defect					
- αα/-$\alpha^{-3.7}$, αα/-$\alpha^{-4.2}$	85	0.14±0.40	22.2±7.1	-	-
- αα/α^{CS}α	14	0.48±0.53	18.4±3.7	-	-
Two α-globin gene defect					
- -$\alpha^{-3.7}$/-$\alpha^{-3.7}$	4	3.7±0.5	19.9±3.5	-	-
- -$\alpha^{-3.7}$/ α^{CS}α	1	6.1	21.4	-	-
- αα/- - ^{SEA}	27	6.16±1.94	24.4±5.8		
Deletional Hb H disease (- - ^{SEA}/-$\alpha^{-3.7}$)	5	25.2±1.8	24.5±4.6	-	-
Non-deletional Hb H disease (- - ^{SEA}/ α^{CS}α)	2	33.5, 39.0	27.3, 15.3	-	-
Hb Q-Thailand carrier (αα/-$\alpha^{Q\text{-Thailand}}$)	2	0, 0.6	11.9, 16.7	-	Q5.3/Qγ22.2, Q6.3/Qγ18.7
Normal α-globin gene and Hb E carrier (αα/αα, β/β^{E})	41	0.06±0.24	13.2±3.9	1.9±1.0	-
Single α-globin gene defect and Hb E carrier					
- αα/-$\alpha^{-3.7}$, β/β^{E}	14	0.03±0.11	13.7±4.5	1.9±0.9	-
- αα/α^{CS}α, β/β^{E}	5	0.7±0.9	15.7±5.6	1.6±1.1	-
Two α-globin gene defect and Hb E carrier					
- αα/-- ^{SEA}, β/β^{E}	1	4.2	17.1	1.8	-
- -$\alpha^{-3.7}$/ α^{CS}α, β/β^{E}	1	12.9	11.9	0.5	-
Deletional Hb H disease and Hb E carrier (- - ^{SEA}/-$\alpha^{-3.7}$, β/β^{E})	1	23.8	8.6	1.0	-
Non-deletional Hb H disease and Hb E carrier (-- ^{SEA}/ α^{CS}α, β/β^{E})	1	29.4	17.0	2.1	-
Hb E- β-thalassemia (αα/αα, β^{0}/β^{E})	1	-	-	2.2	-

Values are presented as means±SD or as raw data where appropriate. CS: Hb Constant Spring, SEA: Southeast Asian deletion

Table 3 Hematologic parameters in correlation with genotypes

Genotype	Number (425)	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	RBC ($x10^{12}$/L)
Normal α-globin gene (αα/αα)	266	15.7±1.4	47.3±4.6	106.0±4.2	35.3±1.6	4.5±0.4
Single α-globin gene defect						
- αα/-$\alpha^{-3.7}$, αα/-$\alpha^{-4.2}$	66	15.4±1.4	47.0±4.3	98.4±4.4	32.3±1.6	4.8±0.4
- αα/α^{CS}α	13	14.2±1.5	43.0±3.8	100.0±2.4	33.0±0.8	4.3±0.4
Two α-globin gene defect						
- -$\alpha^{-3.7}$/-$\alpha^{-3.7}$	2	13.4, 13.8	43.0, 45.7	86, 91	27.0, 27.5	5.0, 5.0
- -$\alpha^{-3.7}$/ α^{CS}α	1	14.4	44.2	85	27.6	5.2
- αα/- - ^{SEA}	20	14.4±1.4	45.7±4.4	87.8±3.0	27.6±0.8	5.2±0.5
Hb H disease						
- - - ^{SEA}/-$\alpha^{-3.7}$	4	11.9±0.5	39.8±2.7	75.5±4.0	22.6±0.7	5.3±0.1
- - - ^{SEA}/ α^{CS}α	2	12.5, 14.3	42.9, 48.6	80, 84	23.4, 24.5	5.3, 5.8
Hb Q-Thailand carrier (αα/-$\alpha^{Q\text{-}Thailand}$)	2	15.3, 16.7	45.8, 50.4	96, 96	31.9, 31.9	4.8, 5.2
Normal α-globin gene and Hb E carrier (αα/αα, β/β^E)	31	15.8±1.4	47.8±4.3	104.1±4.5	34.4±1.6	4.6±0.4
Single α-globin gene defect and Hb E carrier						
- αα/-$\alpha^{-3.7}$, β/β^E	9	14.4±1.5	43.5±4.3	95.8±5.3	31.7±2.3	4.6±0.4
- αα/α^{CS}α, β/β^E	4	14.4±0.5	43.5±1.5	97.0±3.5	32.0±1.4	4.5±0.3
Two α-globin gene defect and Hb E carrier						
- αα/-- ^{SEA}, β/β^E	1	13.5	43.3	84	26.2	5.1
- -$\alpha^{-3.7}$/ α^{CS}α, β/β^E	1	12.8	39.8	95	30.4	4.2
Deletional Hb H disease and Hb E carrier (- - ^{SEA}/-$\alpha^{-3.7}$, β/β^E)	1	12.4	42.0	77	22.7	5.5
Non-deletional Hb H disease and Hb E carrier (-- ^{SEA}/ α^{CS}α, β/β^E)	1	10.8	36.4	84	25.0	4.3
Hb E- β-thalassemia αα/αα, β^0/β^E	1	15.4	46.0	103	34.3	4.5

Hb: hemoglobin, Hct: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, RBC: red blood cell count

Twenty-one samples were excluded from hematologic analysis for the following reasons: 9 congenital anomalies, 8 infections requiring antibiotics, 2 maternal class A1 diabetes mellitus, and one each of Rh (D) incompatibility and maternal hepatitis B virus carrier. One hundred and twenty samples were further excluded because of delayed analysis after 48 hours. The hematologic parameters seen in each genotype are shown in Table 3.

Using an MCV $\leq$ 95 fL, MCH $\leq$30 pg, or Hb Bart's level $\geq$ 3.0% as a cutoff point to screen for α-thalassemia 1, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) are shown in Table 4.

Table 4 Sensitivity, specificity, PPV, and NPV using an MCV $\leq$ 95 fL, MCH $\leq$30 pg, or Hb Bart's level $\geq$ 3.0% as a cutoff point to screen for α-thalassemia 1

Cutoff point	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Bart's level $\geq$ 3.0%	100	98.9	82.4	100
MCV $\leq$ 95 fL	100	92.4	41.2	100
MCH $\leq$ 30 pg	100	98.0	72.4	100

An additional band at Hb A_2 position was seen in 65 samples. All tested positive for Hb E mutation. Two Hb Q-Thailand carriers had two additional Hb fractions apart from Hb F and Hb A, one at Hb Q position and another larger fraction cathodal to Hb Q. They were confirmed to carry the mutation by PCR.

Discussion

This report demonstrates that IEF is a useful method for neonatal screening of thalassemia and hemoglobinopathies. High prevalence of thalassemia in the study population allowed for a diversity of laboratory findings. The allele frequencies were comparable to previous studies where α-thalassemia 2 was highly prevalent, although the frequency of α-thalassemia 1 was lower.[3,4]

For α-thalassemia, the visualization of Hb Bart's depicted α-thalassemia carriers and Hb H diseases. Percentage of Hb Bart's increased with the number of mutated α-globin allele and could distinguish unambiguously among normal individuals, carriers of two α-globin gene defects, and Hb H diseases. In addition, higher Hb Bart's level was seen in non-deletional Hb H diseases compared to deletional Hb H diseases. Hb Bart's level was more than 3.0% in all α-thalassemia 1 carriers. This level can be used as a cutoff point for screening with high sensitivity and specificity. Similarly, Hb Bart's level of more than 20% can be used to identify Hb H disease.

On the other hand, the findings in carriers of single α-globin gene mutation were less clear. The Hb Bart's percentage in this group varied from nil to a small amount where a faint band of Hb Bart's (up to 1.0%) was also seen in individuals without detectable common α-globin gene mutations. This finding agreed with previous studies where a minimal amount of Hb Bart's might be found in individuals with apparently normal α-globin genes.[14,19]

One limitation to this study was that other less common α-globin gene mutations such as Hb Pakse[6], which might cause an elevation of Hb Bart's, were not looked for. Another less common α-thalassemia 1, Thai-deletion[6], was not likely to be present, as all cases with high Hb Bart's had two or three known α-globin gene defects.

Hb Q-Thailand, or Hb Mahidol, or Hb G-Taichung ($-\alpha^{74\ G\text{-}C}$), was firstly reported in 1970.[30-32] Compound heterozygosity of α-thalassemia 1 and Hb Q-Thailand ($- -/-\alpha^{74\ G\text{-}C}$) resulted in the Hb H phenotype. Herein an Hb pattern in two newborns who were carriers of Hb Q-Thailand was demonstrated. Apart from Hb F, Hb A, and a small amount of Hb Q as seen in an adult carrier, an additional band was present cathodal to Hb Q, which was likely a combination of γ-globin and the α-globin variant. Hb Bart's was seen in a small amount in one case and absent in the other, in the same range as carriers of single-α gene defect.

Hb A_2 usually does not present or presents in a very small amount in newborns. The presence of an Hb fraction at the Hb A_2 position in Thai newborns usually indicates Hb E.[20] In this study, all samples with a band at the Hb A_2 position were confirmed Hb E carriers. One case with Hb E and absent Hb A was a compound heterozygosity of β-thalassemia and Hb E. A similar Hb pattern of FE was also seen in Hb E homozygotes. Lorey et al demonstrated that the Hb E level in newborns with Hb E-β-thalassemia was markedly lower than in newborns with homozygous Hb E.[33] Our patient's Hb E level of 2.2% agreed with this previous report.

Red blood cell indices can discriminate between normal individuals, carriers of two α-globin gene defects, and Hb H diseases. MCV $\leq$ 95 fL or MCH $\leq$ 30 pg can be used to screen for α-thalassemia 1 with high sensitivity and specificity. MCV $\leq$ 85 fL or MCH $\leq$ 26 pg suggest a diagnosis of Hb H disease.

Interestingly, carriers of two α-globin gene mutations also have slightly lower Hb levels and individuals with Hb H disease have lower Hb levels than normal. A recent report by Srisupundit et al also demonstrated differences in Hb, MCV, MCH, and MCHC among Hb Bart's diseases, α-thalassemia 1 carriers, and normal fetuses at mid-gestation [34]. This portrayed the crucial role of α-globin genes from the fetal period. On the contrary, as γ-globin genes are the main functioning non α-globin genes during the fetal and neonatal periods, mutations of β-globin genes are expected to cause less hematological changes. As seen in this study, mean Hb and Hct were not different between Hb E carriers and non-carriers, and the differences of MCV and MCH were minimal. Lower Hb A levels were seen in newborns who were carriers for β-thalassemia.[11,13] This subject was beyond the scope of this study. However, it was observed that newborns who were carriers for Hb E had lower levels of Hb A than the non-carriers.

Another interesting finding in the study concerns the effect of concurrent Hb E on Hb H disease. Hb H disease patients with concurrent Hb E beyond infancy were known to have lower level of Hb H and Bart's than those without Hb E.[6] From the current study, two newborns who had Hb H disease and concurrent Hb E had only slightly lower levels of Hb Bart's than other newborns with Hb H disease. This also demonstrated a lesser effect of the β-globin gene mutation during the newborn period.

A weak point in this study was a possible effect of Hb degradation, as IEF analysis was performed in whole blood samples that were kept up to four weeks. Wajcman et al reported an accurate identification of several Hb variants by IEF from dried blood samples after prolonged storage.[35] Roa et al performed serial analysis by HPLC of dried blood

specimens stored at room temperature and reported that the loss of Hb A was 22% at 10 weeks of storage. With the presence of Hb F, the Hb A degradation was accelerated. It was concluded that Hb quantitation by HPLC was accurate within three weeks of storage and identification could be achieved at six weeks.[36]

Compared with a recently reported neonatal screening of thalassemia and Hb variants by HPLC by Tritipsombat et al[20], IEF demonstrated a similarly high discriminating ability. It was notable that the levels of Hb Bart's and Hb E determined by IEF were slightly less than those obtained by HPLC.[19,20] This could be due to the differences between the methods, though the effects of Hb degradation may also be a factor. Hb Bart's ranges by IEF were more comparable to those obtained by cellulose acetate gel electrophoresis.[15,16,18]

In summary, IEF screening for α-thalassemia and Hb variants in newborns correlates well with the globin genotypes. The method can clearly differentiate between carriers of two α-globin gene mutations, Hb H diseases, Hb Q-Thailand carriers, Hb E carriers, and an Hb E-β-thalassemia compared to normal individuals. For carriers of single α-globin gene mutation, Hb Bart's may be either absent or present in a small amount and is therefore not reliable for screening. MCV and MCH are also useful for differentiating two α-globin gene defects and Hb H diseases, but not for Hb E carriers or carriers of single α-globin gene mutation.

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Study 3

Prevalence and molecular characterization of glucose-6-phosphate dehydrogenase deficiency in northern Thailand

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common inherited enzymopathy.[1] As G6PD deficiency protects against malarial infection, the prevalence is high in endemic area of malaria including southeast-Asia and Thailand.[1,2] G6PD enzyme catalyzes a production of nicotinamide adenine dinucleotide phosphate (NADPH) in pentose phosphate pathway. NADPH is essential for the conversion of glutathione to a reduced form, which functions as an antioxidant. G6PD-deficient red blood cells are subjected to oxidation injury and premature hemolysis.[3]

Individuals with G6PD deficiency are usually asymptomatic, but may present with acute hemolysis when exposed to oxidizing agents. They are also at increased risk of neonatal hyperbilirubinemia. A small subset of G6PD-deficiency presents with chronic hemolytic anemia.[3]

G6PD deficiency has an X-linked recessive inheritance. Symptomatic patients are mostly hemizygous males, and also the less common homozygous females. Some heterozygous females may have a decreased level of G6PD enzyme in the intermediate-deficient range, a phenomenon attributed to the skewed inactivation of X-chromosome as described by Lyon in 1961.[4] G6PD-deficient heterozygous females may experience acute hemolysis and are also at increased risk for neonatal hyperbilirubinemia.[5-7]

G6PD deficiency is common in Thai population with the prevalence rage of 3-18% in males.[8] G6PD Viangchan (871G>A) is reportedly the most common variant in the population in the central and southern Thailand.[9,10] G6PD Mahidol (487G>A) was also reported to be the most common variant in the south.[11,12] Other common mutations include G6PD Kaiping (1388G>A), G6PD Canton (1376G>T), G6PD Union (1360C>T) and G6PD Chinese-5 (1024C>T).[9,10] The molecular features are similar to those in Laotians[13], Burmese[14,15], Cambodians[16,17], Malaysians[18,19], southern Vietnamese[20], and southern Chinese[21,22]. The finding suggests common ancestral origin of Thais with other population in southeast-Asia.

Northern Thailand is bordered by Myanmar and Laos, and is close to the southern part of China. This study took place in Chiang Mai, the major province of northern Thailand. The population comprises mainly Thais and assimilated Chinese. In the mountainous areas around Chiang Mai, there are several tribal groups including Karen, Lahu, Hmong, Lisu, Akha, Yao, Lua, Palong and Tai.

Herein we report the prevalence of G6PD deficiency and molecular characterization of the G6PD variants in northern Thai newborns. The study shows that G6PD deficiency has a high prevalence of 17% among male newborns. The mutations are heterogeneous. G6PD Mahidol, G6PD Kaiping, G6PD Canton, and G6PD Viangchan are the most common mutations.

Materials and Methods

Neonatal cord blood screening for G6PD deficiency was conducted. The study protocol was approved by the Institutional Ethics Committee. Informed consent was obtained from the mothers. Full-term newborns (gestational age 37-42 weeks) of mothers with uneventful pregnancy who were born at Chiang Mai University Hospital were included. After delivery of the newborn, umbilical cord blood was collected; 7 mL in ACD for G6PD assay and 5 mL in EDTA for DNA study. The blood samples were kept at 4^{o}C until analysis. The samples were tested for G6PD enzyme level. The samples which were G6PD deficient were further tested for G6PD mutations.

G6PD assay was performed according to the WHO method within 7 days of blood collection.[23] Average G6PD level in cord blood from male newborns was 12.5±2.3 IU/g Hb. Complete and intermediate deficiency of G6PD enzyme were defined by a level of less than 1.5 and 1.5-8.0 IU/g Hb respectively.

Genomic DNA was extracted from leukocytes by Chelex method.[24] Six common G6PD mutations previously reported in Thailand were tested. G6PD Viangchan (871G>A), G6PD Mahidol (487G>A), G6PD Kaiping (1388G>A), G6PD Canton (1376G>T), G6PD Union (1360C>T) and G6PD Chinese-5 (1024C>T) were tested using polymerase chain reaction-restriction fragment length analysis as previously described.[10, 25]

Results

Ninety of five hundred and sixty-six newborns (16%) had G6PD deficiency. The prevalence in male newborns was 17% (48 of 289 male newborns). The prevalence of female newborns having an intermediate deficiency and a complete deficiency of G6PD enzyme was 13% (37 of 277 female newborns) and 1.8% (5 of 277 female newborns) respectively.

The type of G6PD mutations are shown in table 1. G6PD Mahidol, G6PD Kaiping, G6PD Canton and G6PD Viangchan are the most common mutations. These four mutations combined comprise about 70% of G6PD mutations in the population.

Table 1 G6PD mutations in northern Thais

Level of G6PD deficiency	Number of cases	Number of alleles	G6PD mutations						
			Mahidol	Kaiping	Canton	Viangchan	Union	Chinese-5	Unknown
Complete-deficient males	48	48	10	10	6	5	5	2	10
Intermediate-deficient females	37	37	9	6	6	4	2	0	10
Complete-deficient females	5	10	-	1	3	4	-	-	2
Total, n (%)	90	95 (100)	19 (20)	17 (18)	15 (16)	13 (14)	7 (7)	2 (2)	22 (23)

Discussion

The prevalence of G6PD deficiency is high in northern Thai population. Interestingly, as high as 1.8% of female newborns had deficiency of G6PD enzyme in the homozygous range. The finding is comparable with the calculated probability from the allele frequency of G6PD variants (q) by Hardy-Weinberg principle. The allele frequency in our population is 0.1661, portion of G6PD deficient homozygous female equals to q^2, therefore the calculated prevalence is ~2.8% in females.

In northern Thai newborns, G6PD Mahidol, G6PD Kaiping, G6PD Canton and G6PD Viangchan are the most common mutations. G6PD Mahidol is the most common mutation, although the prevalence of these four mutations are about the same (range 14-20%). This finding is similar to the studies in southern Thailand.[11,12] However, it is different from recent studies which reported G6PD Viangchan as the most common G6PD variant, in central Thai population by Nuchprayoon et al[10] and southern Thai population by Laosombat et al.[9]

G6PD Mahidol is the most common variant in Myanmar.[14,15] The geographic proximity between Chiang Mai and Myanmar may explain the genetic similarity between the study population and the Burmese.

G6PD Kaiping and G6PD Canton are common in southern China and Taiwan.[21,22,26] These two mutations are prominent in the study population, supporting the assimilation of the Chinese to northern Thailand.

The presence of G6PD Mahidol, G6PD Kaiping, G6PD Canton and G6PD Viangchan point toward a common ancestry of the northern Thais with other southeast-Asian and southern Chinese population. The higher proportion of G6PD Mahidol, G6PD Kaiping, G6PD Canton in our population as opposed to G6PD Viangchan being the commonest mutations in the other parts of Thailand suggests a significant genetic drift from China and Myanmar into the region.

This study confirms the high prevalence of G6PD deficiency, and describes the new findings on characteristics of *G6PD* mutations in northern Thailand. The G6PD variants are heterogeneous and share similarity with surrounding regions, especially with Myanmar and Southern China.

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ผลผลิตที่ได้จากโครงการ

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Cord blood screening for α-thalassemia and hemoglobin variants by isoelectric focusing in northern Thai neonates: Correlation with genotypes and hematologic parameters

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Isoelectric focusing

ABSTRACT

We describe the screening of newborns for thalassemia and Hb variants by using isoelectric focusing (IEF) in a population from northern Thailand where hemoglobinopathies are highly prevalent. The report focuses on findings of α-thalassemia, Hb E, and other hemoglobin variants, and their correlation with genotypes and hematologic parameters. Two-hundred and seven out of 566 newborns (36.6%) had thalassemia genes or Hb variants. Seventeen different genotypes were found. Nine cases (1.6%) of Hb H disease (five deletional Hb H diseases, two Hb H/Constant Spring diseases, one deletional Hb H disease/Hb E, carrier and one Hb H/Constant Spring disease/Hb E carrier) and one Hb E-β-thalassemia were identified. IEF could clearly distinguish Hb H diseases and carriers of two α-globin gene defects from normal individuals according to the presence of Hb Bart's and its percentage. For carriers of a single α-globin gene defect, Hb Bart's was either absent or present in a small amount and was therefore not reliable for screening. The presence of an additional band at the Hb A_2 position in the newborns signified an Hb E carrier. One case of an absent Hb A and a presence of Hb E was identified as Hb E-β-thalassemia. Two Hb Q-Thailand carriers were seen with two additional Hb fractions, presumably combinations of γ-globin and β-globin with the α-globin variant. Newborns with Hb H disease had lower Hb, MCV, and MCH levels than normal. MCV and MCH were also useful for differentiation of carriers of two α-globin gene defects, but not for carriers of Hb E or single α-globin gene defect. IEF was a reliable method for neonatal cord blood screening for α-thalassemia and Hb variants.

Introduction

Newborn screening for hemoglobinopathies aims to detect thalassemia syndromes and hemoglobin (Hb) variants with significant effects to health, which would allow prevention of complications, early management, and genetic counseling [1,2]. Northern Thailand is among areas in the world where both α- and β-hemoglobinopathies are highly prevalent and interactions between different thalassemia genes lead to a diversity of thalassemia genotypes [3–7]. Through prenatal screening and diagnosis, severe thalassemia diseases, including homozygous α-thalassemia 1 causing Hb Bart's hydrops fetalis, homozygous β-thalassemia, and Hb E-β-thalassemia are usually detected prenatally [8]. On the other hand, Hb H disease, a condition with mild to moderate anemia caused by defects of three α-globin genes leaving only one intact gene, is usually not searched for. Although generally of mild severity, patients with Hb H disease may develop acute hemolysis necessitating blood transfusion when they have fever. Some are transfusion dependent, and there have been reports of hydrops fetalis associated with non-deletional Hb H disease [9,10]. The prevalence of Hb H disease is as high as 1:65 in the northern Thai population [3]. Early identification from the newborn period will facilitate proper parental education, and prevent complications, unnecessary investigation, or iron treatment. Identification of carriers of thalassemia or Hb variants, especially for Hb E which is common in the population, will also be useful for family genetic counseling.

Several Hb analytic methods have been used for this purpose, including starch gel electrophoresis, cellulose acetate gel electrophoresis, isoelectric focusing (IEF), and high pressure liquid column chromatography (HPLC) [11–20]. Currently IEF and HPLC are widely used. These methods can detect α-thalassemia carriers and diseases, β-thalassemia diseases and most Hb variants. Identification of β-thalassemia carriers requires molecular study, although lower Hb A

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amount has been shown to correlate with β-thalassemia carriers and may be used as a primary screen [11,13]. Identification of α-thalassemia depends on the presence of Hb Bart's, which reflects an imbalance of α-globin and γ-globin synthesis [15–21]. The findings are seen most distinctively during the fetal and neonatal periods, when the γ-globin production is active. Hb variants are characterized by the presence of a varying amount of Hb with different electrophoretic properties than the normal pattern of Hb F and Hb A in newborns.

IEF is an electrophoretic separation of different proteins according to their isoelectric points (pIs). A pH gradient is created by applying electrical current to an agarose gel containing low molecular weight amphoteric molecules with varying pIs. Each Hb focuses at the pH position which corresponds to its pI [22]. IEF of Hbs in normal newborns typically shows Hb F and Hb A. Acetylated Hb F (Hb Fac), which is a derivative of Hb F, is also usually seen at approximately 10% of Hb F [21]. Quantitation of each Hb fractions can be further performed by a densitometric analysis of the gel. IEF has the advantage of high discriminatory ability [12,21,23]. The method is also highly sensitive, which is desirable for newborn screening when some Hb variants are present in small amounts. However, the method requires technical skills and reference values of different thalassemia syndromes, and Hb variants prevalent in the target population need to be established.

Herein, we report on newborn screening for thalassemia syndromes and Hb variants using IEF in a northern Thailand population where hemoglobinopathies are highly prevalent. This report focuses on findings of α-thalassemia, Hb E, and other hemoglobin variants, and their correlation with genotypes and hematologic parameters.

Materials and methods

The study protocol was approved by the institutional ethics committee. Informed consent was obtained from the mothers. Five-hundred and sixty-six full-term newborns (gestational age 37–42 weeks) of mothers with uneventful pregnancy who were born at Chiang Mai University Hospital were included. After delivery of the newborn, the umbilical cord was cleaned with povidone iodine, and an 18-gauge needle was inserted into an umbilical vein to collect 5 ml of blood samples in EDTA. The blood samples were kept at 4 °C until analysis.

The Hb level and red cell indices were determined with an electronic cell counter (Beckman Coulter $A^C \cdot T$ 5diff Hematology Analyzer) within 48 h of blood collection. IEF was performed within 4 weeks of blood collection using a Resolve Hemoglobin Kit (PerkinElmer Life and Analytical Sciences, Turku, Finland) as per the manufacturer's recommendation. After electrophoresis, the gels were scanned and analyzed by IsoScan® software (PerkinElmer Life and Analytical Sciences). Firstly the Hb fractions were outlined and percentages were automatically determined by the software program. Then the results were re-examined by two staff members for faint bands that were not automatically detected. The faint bands were outlined manually.

Genomic DNA was extracted from leukocytes by Chelex method [24]. Alpha-globin gene mutations were identified by polymerase chain reaction for three α-globin gene deletions: Southeast-Asian ($--^{SEA}$), 3.7 kb ($-\alpha^{-3.7}$) and 4.2 kb ($-\alpha^{-4.2}$) deletions; and a point mutation at termination codon causing Hb Constant Spring (Hb CS, $\alpha^{142\ T\text{-}C}\alpha$) by methods described previously [25–27].

Amplification refractory mutation system (ARMS)-polymerase chain reaction (PCR) for Hb Q-Thailand ($-\alpha^{74\ G\text{-}C}$) mutation was performed in two samples to confirm the diagnosis of Hb Q-Thailand carrier. Three primers were used: Hb Q-S (5′-AGT ATG GTG CGG AGG CCC TGG AGA-3′), a common upstream primer; Hb Q-N (5′-CAG GGC GGA CAG CGC GTT GGG CAT GTC GTC-3′); and Hb Q-M1 (5′-CAG GGC GGA CAG CGC GTT GGG CAT GTC GAG-3′). The Hb Q-N and Hb Q-S produced a 293 bp fragment from normal templates and the Hb Q-M1 and Hb Q-S produced a 293 bp fragment from mutational templates. Two primers were used as controls: AE12 (5′-TGC AAT CAT TCG TCT GTT TCC C-3′) and SE12 (5′-AGA AGA GCC AAG GAC AGG TAC G). The control product was 660 bp in length. The PCR mixture (25 μl) contained 8 μl DNA, 0.1 μmol/l of each primer, 200 μmol/l dNTPs, 1 unit of Platinum® Taq DNA polymerase (Qiagen, Germany), in 1× PCR buffer (20 mmol/l Tris–HCl (pH 8.4) and 50 mmol/l KCl), and 1.5 mmol/l $MgCl_2$. After a 15-min initial denaturation at 95 °C, 35 cycles of 94 °C 60 s, 56 °C 60 s and 72 °C 2 min were performed on a GeneAmp PCR system 9700 (Perkin Elmer, CT, USA). The PCR products were analyzed by electrophoresis on a 2% agarose gel containing 0.5 mg/ml of ethidium bromide. The DNA bands were detected by UV light and documented using a Bio-Rad Gel Doc 1000 system.

Identification of Hb E ($\beta^{26\ G\text{-}A}$) mutation was performed by an ARMS-PCR in 65 samples with the presence of an additional band at the Hb A_2 position to confirm the diagnosis of Hb E carrier. The method was described previously [28]. ARMS-PCR for β-thalassemia and real-time PCR for β-globin gene deletion ($\beta^{3.5\ kb\ del}$) were performed on a case with Hb E-β-thalassemia by methods described previously [28,29].

Records of newborn admissions were reviewed. Factors that may interfere with hematologic parameters, such as infections, maternal diabetes mellitus, Rh(D) compatibility, or congenital anomalies, were searched for. If any of these factors were present, only the results of globin genotypes and IEF were included for analysis; hematologic parameters were excluded.

Hematologic and hemoglobin parameters were summarized for each α-globin and β^E genotypes and were expressed as means and standard deviations.

Results

Two-hundred and seven out of 566 newborns (36.6%) had thalassemia genes or Hb variants. Alpha-thalassemia 2 (3.7 and 4.2 kb deletions) carrier was the most common genotype (15.0%), followed by Hb E carrier (7.2%), α-thalassemia 1 (Southeast Asian deletion) carrier (4.9%), Hb CS carrier (2.7%), and Hb Q-Thailand carrier (0.4%). Nine newborns (1.6%) had Hb H disease: five cases of deletional Hb H disease ($--^{SEA}/-\alpha^{3.7}$), two non-deletional Hb H disease ($--^{SEA}/\alpha^{CS}\alpha$), and two double heterozygous Hb H disease and Hb E carrier. One was a compound heterozygote for Hb E-β-thalassemia ($\beta^{3.5\ kb\ del}/\beta^{26\ G\text{-}A}$). Overall, there were 17 thalassemia and Hb variant genotypes. Their prevalence is shown in Table 1. The Hb patterns seen in each genotype are shown in Table 2.

Twenty-one samples were excluded from hematologic analysis for the following reasons: nine congenital anomalies, eight infections

Table 1
Genotypes of thalassemia and hemoglobin variants.

Genotype	Number (%)	
Single α-globin gene defect	$\alpha\alpha/-\alpha^{-3.7}$	80 (14.1)
	$\alpha\alpha/-\alpha^{-4.2}$	5 (0.9)
	$\alpha\alpha/\alpha^{CS}\alpha$	15 (2.7)
Two α-globin gene defect	$-\alpha^{-3.7}/-\alpha^{-3.7}$	4 (0.7)
	$-\alpha^{-3.7}/\alpha^{CS}\alpha$	1 (0.2)
	$\alpha\alpha/--^{SEA}$	28 (4.9)
Hb H disease	$--^{SEA}/-\alpha^{-3.7}$	5 (0.9)
	$--^{SEA}/\alpha^{CS}\alpha$	2 (0.4)
α-Globin variant	$\alpha\alpha/-\alpha^{Q\text{-}Thailand}$	2 (0.4)
Normal α-globin gene and Hb E carrier	$\alpha\alpha/\alpha\alpha, \beta/\beta^E$	41 (7.2)
Single α-globin gene defect and Hb E carrier	$\alpha\alpha/-\alpha^{-3.7}, \beta/\beta^E$	14 (2.4)
	$\alpha\alpha/\alpha^{CS}\alpha, \beta/\beta^E$	5 (0.9)
Two α-globin gene defect and Hb E carrier	$\alpha\alpha/--^{SEA}, \beta/\beta^E$	1 (0.2)
	$-\alpha^{-3.7}/\alpha^{CS}\alpha, \beta/\beta^E$	1 (0.2)
Hb H disease and Hb E carrier	$--^{SEA}/-\alpha^{-3.7}, \beta/\beta^E$	1 (0.2)
	$--^{SEA}/\alpha^{CS}\alpha, \beta/\beta^E$	1 (0.2)
Hb E-β-thalassemia	$\alpha\alpha/\alpha\alpha, \beta^0/\beta^E$	1 (0.2)
Normal α-globin gene	$\alpha\alpha/\alpha\alpha$	359 (63.4)
Total		566 (100)

Table 2
Hemoglobin patterns in correlation with genotypes.

Genotype	Number (562)	Hb Bart's (%)	Hb A (%)	Hb A2/E (%)	Hb variant (%)
Normal α-globin gene (αα/αα)	357	0.02 ± 0.12	20.7 ± 6.2	–	–
Single α-globin gene defect					
αα/−α$^{-3.7}$, αα/−α$^{-4.2}$	85	0.14 ± 0.40	22.2 ± 7.1	–	–
αα/α^{CS}α	14	0.48 ± 0.53	18.4 ± 3.7	–	–
Two α-globin gene defect					
−α$^{-3.7}$/−α$^{-3.7}$	4	3.7 ± 0.5	19.9 ± 3.5	–	–
−α$^{-3.7}$/α^{CS}α	1	6.1	21.4	–	–
αα/– –^{SEA}	27	6.16 ± 1.94	24.4 ± 5.8		
Deletional Hb H disease (– –^{SEA}/−α$^{-3.7}$)	5	25.2 ± 1.8	24.5 ± 4.6	–	–
Non-deletional Hb H disease (– –^{SEA}/α^{CS}α)	2	33.5, 39.0	27.3, 15.3	–	–
Hb Q-Thailand carrier (αα/−α$^{Q\text{-}Thailand}$)	2	0, 0.6	11.9, 16.7	–	Q5.3/Qγ22.2, Q6.3/Qγ18.7
Normal α-globin gene and Hb E carrier (αα/αα, β/β^{E})	41	0.06 ± 0.24	13.2 ± 3.9	1.9 ± 1.0	–
Single α-globin gene defect and Hb E carrier					
αα/−α$^{-3.7}$, β/β^{E}	14	0.03 ± 0.11	13.7 ± 4.5	1.9 ± 0.9	–
αα/α^{CS}α, β/β^{E}	5		15.7 ± 5.6	1.6 ± 1.1	–
Two α-globin gene defect and Hb E carrier					
αα/– –^{SEA}, β/β^{E}	1	4.2	17.1	1.8	–
α$^{-3.7}$/α^{CS}α, β/β^{E}	1	12.9	11.9	0.5	–
Deletional Hb H disease and Hb E carrier (– –^{SEA}/−α$^{-3.7}$, β/β^{E})	1	23.8	8.6	1.0	–
Non-deletional Hb H disease and Hb E carrier (– –^{SEA}/α^{CS}α, β/β^{E})	1	29.4	17.0	2.1	–
Hb E-β-thalassemia (αα/αα, β^{0}/β^{E})	1	–	–	2.2	–

Values are presented as means ± SD or as raw data where appropriate. CS: Hb Constant Spring, SEA: Southeast Asian deletion.

Table 3
Hematologic parameters in correlation with genotypes.

Genotype	Number (425)	Hb (g/dl)	Hct (%)	MCV (fl)	MCH (pg)	RBC (×10^{12}/l)
Normal α-globin gene (αα/αα)	266	15.7 ± 1.4	47.3 ± 4.6	106.0 ± 4.2	35.3 ± 1.6	4.5 ± 0.4
Single α-globin gene defect						
αα/−α$^{-3.7}$, αα/−α$^{-4.2}$	66	15.4 ± 1.4	47.0 ± 4.3	98.4 ± 4.4	32.3 ± 1.6	4.8 ± 0.4
αα/α^{CS}α	13	14.2 ± 1.5	43.0 ± 3.8	100.0 ± 2.4	33.0 ± 0.8	4.3 ± 0.4
Two α-globin gene defect						
−α$^{-3.7}$/−α$^{-3.7}$	2	13.4, 13.8	43.0, 45.7	86, 91	27.0, 27.5	5.0, 5.0
−α$^{-3.7}$/α^{CS}α	1	14.4	44.2	85	27.6	5.2
αα/– –^{SEA}	20	14.4 ± 1.4	45.7 ± 4.4	87.8 ± 3.0	27.6 ± 0.8	5.2 ± 0.5
Hb H disease						
– –^{SEA}/−α$^{-3.7}$	4	11.9 ± 0.5	39.8 ± 2.7	75.5 ± 4.0	22.6 ± 0.7	5.3 ± 0.1
– –^{SEA}/α^{CS}α	2	12.5, 14.3	42.9, 48.6	80, 84	23.4, 24.5	5.3, 5.8
Hb Q-Thailand carrier (αα/−α$^{Q\text{-}Thailand}$)	2	15.3, 16.7	45.8, 50.4	96, 96	31.9, 31.9	4.8, 5.2
Normal α-globin gene and Hb E carrier (αα/αα, β/β^{E})	31	15.8 ± 1.4	47.8 ± 4.3	104.1 ± 4.5	34.4 ± 1.6	4.6 ± 0.4
Single α-globin gene defect and Hb E carrier						
αα/−α$^{-3.7}$, β/β^{E}	9	14.4 ± 1.5	43.5 ± 4.3	95.8 ± 5.3	31.7 ± 2.3	4.6 ± 0.4
αα/α^{CS}α, β/β^{E}	4	14.4 ± 0.5	43.5 ± 1.5	97.0 ± 3.5	32.0 ± 1.4	4.5 ± 0.3
Two α-globin gene defect and Hb E carrier						
αα/– –^{SEA}, β/β^{E}	1	13.5	43.3	84	26.2	5.1
−α$^{-3.7}$/α^{CS}α, β/β^{E}	1	12.8	39.8	95	30.4	4.2
Deletional Hb H disease and Hb E carrier (– –^{SEA}/−α$^{-3.7}$, β/β^{E})	1	12.4	42.0	77	22.7	5.5
Non-deletional Hb H disease and Hb E carrier (– –^{SEA}/α^{CS}α, β/β^{E})	1	10.8	36.4	84	25.0	4.3
Hb E-β-thalassemia αα/αα, β^{0}/β^{E}	1	15.4	46.0	103	34.3	4.5

Hb: hemoglobin, Hct: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, RBC: red blood cell count.

requiring antibiotics, two maternal class A1 diabetes mellitus, and one each of Rh (D) incompatibility and maternal hepatitis B virus carrier. One-hundred and twenty samples were further excluded because of delayed analysis after 48 h. The hematologic parameters seen in each genotype are shown in Table 3.

Using an MCV ≤ 95 fl, MCH ≤ 30 pg, or Hb Bart's level ≥ 3.0% as a cutoff point to screen for α-thalassemia 1, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) are shown in Table 4.

Table 4
Sensitivity, specificity, PPV, and NPV using an MCV ≤ 95 fl, MCH ≤ 30 pg, or Hb Bart's level ≥ 3.0% as a cutoff point to screen for α-thalassemia 1.

Cutoff point	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Bart's level ≥ 3.0%	100	98.9	82.4	100
MCV ≤ 95 fl	100	92.4	41.2	100
MCH ≤ 30 pg	100	98.0	72.4	100

An additional band at Hb A_2 position was seen in 65 samples. All tested positive for Hb E mutation. Two Hb Q-Thailand carriers had two additional Hb fractions apart from Hb F and Hb A, one at Hb Q position and another larger fraction cathodal to Hb Q. They were confirmed to carry the mutation by PCR.

Discussion

This report demonstrates that IEF is a useful method for neonatal screening of thalassemia and hemoglobinopathies. High prevalence of thalassemia in the study population allowed for a diversity of laboratory findings. The allele frequencies were comparable to previous studies where α-thalassemia 2 was highly prevalent, although the frequency of α-thalassemia 1 was lower [3,4].

For α-thalassemia, the visualization of Hb Bart's depicted α-thalassemia carriers and Hb H diseases. Percentage of Hb Bart's increased with the number of mutated α-globin allele and could distinguish unambiguously among normal individuals, carriers of two

α-globin gene defects, and Hb H diseases. In addition, higher Hb Bart's level was seen in non-deletional Hb H diseases compared to deletional Hb H diseases. Hb Bart's level was more than 3.0% in all α-thalassemia 1 carriers. This level can be used as a cutoff point for screening with high sensitivity and specificity. Similarly, Hb Bart's level of more than 20% can be used to identify Hb H disease.

On the other hand, the findings in carriers of single α-globin gene mutation were less clear. The Hb Bart's percentage in this group varied from nil to a small amount where a faint band of Hb Bart's (up to 1.0%) was also seen in individuals without detectable common α-globin gene mutations. This finding agreed with previous studies where a minimal amount of Hb Bart's might be found in individuals with apparently normal α-globin genes [14,19].

One limitation to this study was that other less common α-globin gene mutations such as Hb Pakse [6], which might cause an elevation of Hb Bart's, were not looked for. Another less common α-thalassemia 1, Thai-deletion [6], was not likely to be present, as all cases with high Hb Bart's had two or three known α-globin gene defects.

Hb Q-Thailand, or Hb Mahidol, or Hb G-Taichung ($-\alpha^{74\ G\text{-}C}$), was firstly reported in 1970 [30–32]. Compound heterozygosity of α-thalassemia 1 and Hb Q-Thailand ($--/-\alpha^{74\ G\text{-}C}$) resulted in the Hb H phenotype. Herein an Hb pattern in two newborns who were carriers of Hb Q-Thailand was demonstrated. Apart from Hb F, Hb A, and a small amount of Hb Q as seen in an adult carrier, an additional band was present cathodal to Hb Q, which was likely a combination of γ-globin and the α-globin variant. Hb Bart's was seen in a small amount in one case and absent in the other, in the same range as carriers of single-α gene defect.

Hb A_2 usually does not present or presents in a very small amount in newborns. The presence of an Hb fraction at the Hb A_2 position in Thai newborns usually indicates Hb E [20]. In this study, all samples with a band at the Hb A_2 position were confirmed Hb E carriers. One case with Hb E and absent Hb A was a compound heterozygosity of β-thalassemia and Hb E. A similar Hb pattern of FE was also seen in Hb E homozygotes. Lorey et al. demonstrated that the Hb E level in newborns with Hb E-β-thalassemia was markedly lower than in newborns with homozygous Hb E [33]. Our patient's Hb E level of 2.2% agreed with this previous report.

Red blood cell indices can discriminate between normal individuals, carriers of two α-globin gene defects, and Hb H diseases. MCV ≤ 95 fl or MCH ≤ 30 pg can be used to screen for α-thalassemia 1 with high sensitivity and specificity. MCV ≤ 85 fl or MCH ≤ 26 pg suggests a diagnosis of Hb H disease.

Interestingly, carriers of two α-globin gene mutations also have slightly lower Hb levels and individuals with Hb H disease have lower Hb levels than normal. A recent report by Srisupundit et al. also demonstrated differences in Hb, MCV, MCH, and MCHC among Hb Bart's diseases, α-thalassemia 1 carriers, and normal fetuses at mid-gestation [34]. This portrayed the crucial role of α-globin genes from the fetal period. On the contrary, as γ-globin genes are the main functioning non-α-globin genes during the fetal and neonatal periods, mutations of β-globin genes are expected to cause less hematological changes. As seen in this study, mean Hb and Hct were not different between Hb E carriers and non-carriers, and the differences of MCV and MCH were minimal. Lower Hb A levels were seen in newborns who were carriers for β-thalassemia [11,13]. This subject was beyond the scope of this study. However, it was observed that newborns who were carriers for Hb E had lower levels of Hb A than the non-carriers.

Another interesting finding in the study concerns the effect of concurrent Hb E on Hb H disease. Hb H disease patients with concurrent Hb E beyond infancy were known to have lower level of Hb H and Bart's than those without Hb E [6]. From the current study, two newborns who had Hb H disease and concurrent Hb E had only slightly lower levels of Hb Bart's than other newborns with Hb H disease. This also demonstrated a lesser effect of the β-globin gene mutation during the newborn period.

A weak point in this study was a possible effect of Hb degradation, as IEF analysis was performed in whole blood samples that were kept up to 4 weeks. Wajcman et al. reported an accurate identification of several Hb variants by IEF from dried blood samples after prolonged storage [35]. Roa et al. performed serial analysis by HPLC of dried blood specimens stored at room temperature and reported that the loss of Hb A was 22% at 10 weeks of storage. With the presence of Hb F, the Hb A degradation was accelerated. It was concluded that Hb quantitation by HPLC was accurate within 3 weeks of storage and identification could be achieved at 6 weeks [36].

Compared with a recently reported neonatal screening of thalassemia and Hb variants by HPLC by Tritipsombut et al. [20], IEF demonstrated a similarly high discriminating ability. It was notable that the levels of Hb Bart's and Hb E determined by IEF were slightly less than those obtained by HPLC [19,20]. This could be due to the differences between the methods, though the effects of Hb degradation may also be a factor. Hb Bart's ranges by IEF were more comparable to those obtained by cellulose acetate gel electrophoresis [15,16,18].

In summary, IEF screening for α-thalassemia and Hb variants in newborns correlates well with the globin genotypes. The method can clearly differentiate between carriers of two α-globin gene mutations, Hb H diseases, Hb Q-Thailand carriers, Hb E carriers, and an Hb E-β-thalassemia compared to normal individuals. For carriers of single α-globin gene mutation, Hb Bart's may be either absent or present in a small amount and is therefore not reliable for screening. MCV and MCH are also useful for differentiating two α-globin gene defects and Hb H diseases, but not for Hb E carriers or carriers of single α-globin gene mutation.

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ภาคผนวก 2

Manuscript

Charoenkwan P, Tantiprabha W, Sirichotiyakul S, Piyamongkol W, Taweephol R, Sanguansermsri T. Risk factors for hyperbilirubinemia in Thai newborns.

Risk factors for hyperbilirubinemia in northern Thai newborns

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Keywords: G6PD deficiency, blood group incompatibility, α-thalassemia, *UGT1A1* polymorphism

Abstract

Objective To determine the incidence and risk factors for neonatal hyperbilirubinemia in northern Thai newborns.
Study design A prospective cohort study of healthy full-term newborns was conducted at Chiang Mai University Hospital. Umbilical cord blood was collected after delivery and tested for ABO blood group, G6PD enzyme level, and α-thalassemia. The samples which were G6PD deficient were further tested for *UGT1A1* variants at nucleotide 211 (G211A) and TATA promoter (TA7). Univariate and multivariate logistic regression analyses were performed for risk factors for neonatal hyperbilirubinemia.
Results Five hundred and forty-three newborns were included into the study. Eighty-seven (16%) newborns had hyperbilirubinemia requiring phototherapy. None required exchange transfusion. Delivery by vacuum extraction, ABO blood group incompatibility and G6PD deficiency were significantly associated with twofold to fourfold increase in odds of neonatal hyperbilirubinemia. Type of feeding, percentage of weight loss, cephalohematoma, α^0-thalassemia carrier and hemoglobin H disease were not associated with neonatal hyperbilirubinemia. *UGT1A1* polymorphisms did not increase the incidence of hyperbilirubinemia in G6PD deficient newborns.
Conclusions The incidence of neonatal hyperbilirubinemia is high in northern Thai newborns. ABO blood group incompatibility and G6PD deficiency which are prevalent in the population, and also delivery by vacuum extraction are confirmed as strong risk factors.

Abstract word count: 200

Abbreviations

AAP	American Academy of Pediatrics
CI	Confidence interval
CS	Constant Spring
G6PD	Glucose-6-phosphate dehydrogenase
Hb	Hemoglobin
NH	Neonatal hyperbilirubinemia
nt	Nucleotide
OR	Odds ratio
SLCO1B1	Solute carrier organic anion transporter family member 1B1
UGT1A1	Uridine diphosphoglucuronosyl transferase 1A1

Introduction

Early identification and management of severe neonatal hyperbilirubinemia is important to prevent its most devastating complication, bilirubin encephalopathy. Clinical and genetic factors each play a role in neonatal hyperbilirubinemia.[1] The major risk factors as stated in clinical practice guidelines on hyperbilirubinemia management by American Academy of Pediatrics (AAP) in 2004 include late-preterm gestational age, exclusive breast feeding, blood group incompatibility, hemolytic diseases such as glucose-6-phosphate dehydrogenase (G6PD) deficiency, East Asian race, cephalohematoma or significant bruising and history of previous sibling received phototherapy.[2] Several reported genetic risk factors are polymorphisms of genes modulating bilirubin metabolism, such as the uridine diphosphoglucuronosyl transferase

1A1 (*UGT1A1*) gene that encodes UGT enzyme which involves bilirubin conjugation, and solute carrier organic anion transporter family member 1B1 *(SLCO1B1)* gene that encodes transporting polypeptide which functions in hepatic bilirubin uptake.[1,3-5]

The incidence of non-physiologic hyperbilirubinemia in Asian newborns has been reported to be as high as more than 30%.[6] ABO blood group incompatibilities and G6PD deficiency plus environmental exposure to offending agents are common in the population and may partly explain the high incidence. *UGT1A1* variant at nucleotide (nt) 211 (G211A) and *SLCO1B1* at nt 388 (A388G) are two major variants reported to associate with neonatal hyperbilirubinemia in east Asian newborns.[7-10] *UGT1A1* TATA promoter variant (TA7) which is the risk factor in Caucasians is less common in the population.[8,9]

UGT1A1 polymorphisms are particularly seen in prolonged neonatal hyperbilirubinemia, and especially in exclusively breast-fed newborns.[4,7-9] Also, they are found to be an additive risk factor for neonatal hyperbilirubinemia in G6PD deficient newborns.[5,11,12]

Thailand is a country in Southeast Asia. Reported risk factors of hyperbilirubinemia in Thai newborns are similar to those in East Asian newborns.[13,14] G6PD deficiency and ABO incompatibility which are prevalent in Thais are the main risk factors. Additionally, delivery by vacuum extraction is reportedly a risk factor.[14] *UGT1A1* variant at nt 211, but not *UGT1A1* variant at nt 686 (C686A) or polymorphisms of *SCLO1B1* and *GST* (glutathione S-transferase) genes, were recently reported to be a risk factor for hyperbilirubinemia in Thai newborns.[15]

Hemolytic anemias cause increased bilirubin production, and are therefore a significant cause of neonatal hyperbilirubinemia. In northern Thailand, hemoglobin H (Hb H) disease is a highly prevalent hereditary hemolytic anemia.[16] The condition is caused by deletions with or without point mutations of three out of four normally functioning α-globin genes, leaving one intact gene (– – /– α or – – /α^Tα).[17] The excess β-globin combines to form Hb H which is unstable and has a high affinity for oxygen and is therefore ineffective for oxygen transportation. Patients with Hb H disease generally have mild to moderate anemia. They may present with acute hemolysis triggered by fever or infections. Some reportedly present early in life with neonatal hyperbilirubinemia.[18,19]

There are two types of α-thalassemia carriers, α^0-thalassemia and α^+-thalassemia. The former results from *in-cis* deletion of two α-globin genes and the latter results from deletion or mutation of a single α-globin gene. α^0-thalassemia carriers have hypochromic and microcytic red blood cells. During the newborn period, they have elevated Hb Bart's which demonstrates an imbalance production of alpha- and gamma-globins.[20] They are also shown to have lower Hb level *in utero* from mid-gestation and during the newborn period; this finding demonstrates an important effect of a decreased α-globin production.[20,21] Any additional role of hemolysis on the lower Hb levels in the fetal and newborn periods is unknown. α^+-thalassemia carriers have minimally raised Hb Bart's at birth and are typically asymptomatic.[20]

While Hb H disease is conceivably a risk for neonatal hyperbilirubinemia, the evidence is limited. α^0-thalassemia carrier status was shown to pose no additional risk to newborns with G6PD deficiency.[22] On the contrary, it was shown to be protective in Taiwanese newborns.[23]

The objectives of this study were to determine the incidence of neonatal hyperbilirubinemia and its associated clinical and genetic factors in a cohort study of full-term healthy newborns from northern Thailand. The roles of mode of delivery, type of feeding, degree of weight loss, G6PD deficiency, ABO blood group incompatibilities, α^0-thalassemia carriers and Hb H disease on hyperbilirubinemia were investigated. *UGT1A1*

gene polymorphisms were further explored in cases with G6PD deficiencies for their additive roles on neonatal hyperbilirubinemia.

Materials and Methods

This is a prospective cohort study. The study protocol was approved by the Institutional Ethics Committee. Informed consent was obtained from the mothers. Full-term newborns (gestational age 37-42 weeks) of mothers with uneventful pregnancy who were born at Chiang Mai University Hospital were included. Newborns with known maternal history of Rh(D) incompatibility, diabetes mellitus, and newborns with congenital anomalies, infection requiring antibiotics or significant hemorrhages other than cephalohematoma were excluded from the study.

The umbilical cord blood was collected after delivery of the newborn. The blood samples were kept at 4^{o}C until analysis. The samples were tested for ABO blood group, G6PD enzyme level, and α-thalassemia. The samples which were G6PD deficient were further tested for *UGT1A1* polymorphisms at nt 211 and TATA promoter region.

ABO blood group was determined by a standard technique. ABO incompatibility was defined as maternal blood group O and newborn A or B. G6PD assay was performed according to the WHO method within 7 days of blood collection.[24] Average G6PD level in cord blood from male newborns was 12.5±2.3 IU/g Hb. Complete and intermediate deficiency of G6PD enzyme were defined by a level of less than 1.5 and 1.5-8.0 IU/g Hb respectively. Carriers of Southeast-Asian deletional α^0-thalassemia, the most common α^0-thalassemia in the area, and Hb H disease were diagnosed by polymerase chain reaction and Hb analysis as described previously.[20]

UGT1A1 polymorphisms at nt 211 and TATA promoter region were identified by methods as described previously.[8,25]

Records of newborn admissions were reviewed for clinical features including: gender, gestational age, birth weight and percentage of weight loss from birth within the admission, Apgar score at 5 minutes, delivery method, occurrence and identified causes of neonatal hyperbilirubinemia requiring phototherapy or exchange transfusion. The decision for treatment of neonatal hyperbilirubinemia was up to the pediatric residents and attending neonatologists, who were encouraged to follow the AAP 2004 guideline.[2] Lower threshold of bilirubin level for phototherapy may be used for newborns with undefined risk factors who awaited laboratory results. At our hospital, healthy newborns are usually discharged after blood sampling for thyroid and phenylketonuria screening after 48 hours. Newborns with suspected hyperbilirubinemia are kept in the hospital longer for observation and treatment as needed.

Clinical features were summarized for newborns with and without hyperbilirubinemia. Univariate binary logistic analysis was performed for delivery method, presence of cephalohematoma, type of feeding, percentage of weight loss, G6PD enzyme status, ABO blood group incompatibilities, and α-thalassemia status. Factors that were found to be significantly associated with neonatal hyperbilirubinemia were further evaluated by multivariate binary logistic analysis for adjusted odds ratio and their 95% confidence intervals (CI). Incidence of neonatal hyperbilirubinemia in G6PD deficient newborns was compared between groups with and without *UGT1A1* polymorphisms using Chi-square test. P-values of less than 0.05 were considered statistically significant.

Results

Five hundred and sixty-six newborns were enrolled in the study. Twenty-three were excluded for the following reasons: 9 congenital anomalies, 8 infections requiring treatment with antibiotics, 2 maternal class A1 diabetes mellitus, 2 known significant hemorrhage (1 intracranial hemorrhage and 1 large hematoma), and 1 each of Rh (D) incompatibility and maternal hepatitis B virus carrier.

The clinical features of the included 543 newborns are shown in Table 1. There were 278 (51.2%) male newborns. Mean gestational age was 38.7±1.1 weeks. All had Apgar scores at 5 minutes of not less than 8. Eighty-seven (16%) newborns had hyperbilirubinemia requiring phototherapy. None required exchange transfusion. Causes of neonatal hyperbilirubinemia were identified in 39 (44.8%) newborns: 26 G6PD deficiency, 7 Coombs' positive ABO blood group incompatibility, 3 concurrent G6PD deficiency and ABO blood group incompatibility, 2 cephalohematoma, and 1 Hb H disease. A newborn with deletional Hb H disease who had hyperbilirubinemia had no other known risk factors; he was the first child of the family, born by normal delivery, had a birth weight of 2,700 g, had elevated G6PD level (31.05 u/g Hb). Both mother and child had A, Rh(D) positive blood group. The result of direct and indirect Coombs' tests on the child's blood were negative. All 9 newborns with Hb H disease (5 deletional Hb H disease, 2 Hb H/Hb Constant Spring (CS) disease, 1 AEBart's disease and 1 AEBart'sCS disease) had elevated G6PD levels (24.4±3.0 u/g Hb, range 21.1-31.1 u/g Hb).

Tables 2 and 3 respectively show the odds ratio and 95% CI for factors obtained by univariate and multivariate binary logistic analyses. Delivery by vacuum extraction, ABO blood group incompatibility and G6PD deficiency were associated with development of neonatal hyperbilirubinemia. Cephalohematoma, type of feeding, percentage of weight loss, α^0-thalassemia carrier status and Hb H disease were not associated with neonatal hyperbilirubinemia.

Allele frequencies of *UGT1A1* polymorphisms at nt 211 and TATA promoter region were 0.15 and 0.16 respectively. Neither was associated with neonatal hyperbilirubinemia in G6PD deficient newborns. The results are shown in Table 4.

Discussion

This is a comprehensive study of the roles of clinical and genetic factors in the development of neonatal hyperbilirubinemia in northern Thai newborns. The study shows a high incidence of neonatal hyperbilirubinemia necessitating treatment in the population, and emphasizes the important roles of factors including G6PD deficiency, ABO blood group incompatibilities, and delivery by vacuum extraction, in neonatal hyperbilirubinemia. The study also demonstrates that cephalohematoma, type of feeding, percentage of weight loss, α^0-thalassemia carriers and Hb H disease are not associated with neonatal hyperbilirubinemia. *UGT1A1* polymorphisms common in the population are not an additive risk factor for neonatal hyperbilirubinemia in G6PD deficient newborns.

G6PD deficient newborns were at significantly higher risk for neonatal hyperbilirubinemia. Hemizygous male and homozygous female G6PD-deficient newborns were at higher risk for neonatal hyperbilirubinemia than their heterozygous counterparts with intermediate deficiency of G6PD enzyme (adjusted odds ratio 4.2 and 3.4, respectively). This demonstrated the dosage effect of G6PD deficiency on bilirubin metabolism. This finding agrees with previous studies, and indicates that female carriers

with intermediate deficiency of G6PD enzyme are also at risk and should be monitored for neonatal hyperbilirubinemia.[26,27]

ABO blood group incompatibilities were an important factor for neonatal hyperbilirubinemia. In this study, Coombs' tests were not routinely done, so it was not possible to distinguish the effects of Coombs' positive and negative ABO blood group incompatibilities on the development of neonatal hyperbilirubinemia.

Delivery by vacuum extraction was a risk for neonatal hyperbilirubinemia. The finding agrees with previous reports.[14,28] The effect might be explained by an inevitably higher possibility of soft tissue trauma and hemorrhage associated with the procedure. Justifiably, Caesarian section seemed to be a protective factor, although the difference was not significant. Cephalohematoma was not a risk factor. However the number of events was small.

α^0-thalassemia carriers and Hb H disease were neither a risk nor a protective factor for neonatal hyperbilirubinemia. This suggested negligible or no hemolysis in α^0-thalassemia carriers. However, increased hemolysis was evident in Hb H disease as indirectly indicated by elevated G6PD levels in all 9 Hb H newborns. In spite of this, only 1 had neonatal hyperbilirubinemia. This might imply that only a small number of newborns with Hb H disease experienced hemolysis that was serious enough to lead to neonatal hyperbilirubinemia. This interesting finding needs confirmation in larger Hb H disease population.

UGT1A1 polymorphisms at nt 211 and TATA promoter region were previously reported to associate with higher bilirubin levels and gallstones in Thai Hb E/β-thalassemia patients.[29] Prachukthum *et al* reported that only *UGT1A1* polymorphisms at nt 211 was related to neonatal hyperbilirubinemia in a case-control study of newborns from central Thailand.[15] In this study, the gene frequency of the *UGT1A1* polymorphism at nt 211 polymorphism was slightly higher and that the polymorphism at TATA promoter region was comparable with these previous studies. However, neither polymorphism increased the incidence of neonatal hyperbilirubinemia in G6PD deficient newborns. Our finding agrees with previous studies that *UGT1A1* polymorphisms are not a risk factor for hyperbilirubinemia in the G6PD deficient newborns.[30,31] *UGT1A1* polymorphisms were reported to be an important risk for prolonged hyperbilirubinemia, especially in exclusively breast-fed newborns.[4,7-9] A shorter period of observation and a lower portion of exclusively breast-fed newborns in this study might have masked the effect of the polymorphisms.

Type of feeding was not different between the group with and without neonatal hyperbilirubinemia. However, the exact amount of supplementary feeding in each newborn was difficult to determine. This may have confounded the result in the supplementary group. Percentage of weight loss was used as an indicator of dehydration. It was also found not associated with neonatal hyperbilirubinemia. This again may be confounded by treatment as supplementary feeding was usually given for newborns who lost more than a predetermined percentage of birth weight.

The strength of this study is the large study population and the high prevalence of G6PD deficiency, ABO incompatibility and α-thalassemia, which allow for an identification of the roles of each factor. The weakness of this study is that as we collected the clinical data from hospital records, late-onset hyperbilirubinemia and readmissions to other hospitals might have been missed. Another remark is that the rate of phototherapy may be higher as some newborns were given phototherapy at lower bilirubin threshold while awaiting investigation results.

This study confirms a high incidence of neonatal hyperbilirubinemia in northern Thai population. It substantiates the roles of clinical factors including G6PD enzyme

deficiency, ABO blood group incompatibility, and delivery by vacuum extraction, and refutes the roles of type of feeding, weight loss, α-thalassemia and common *UGT1A1* polymorphisms in combination with G6PD deficiency on neonatal hyperbilirubinemia.

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Table 1: Clinical features of newborns classified by the presence or absence of neonatal hyperbilirubinemia (NH)

Clinical features	**NH**	**Non NH**	**p-value**
Number, n (%)	87 (16.0)	456 (84.0)	-
Male gender, n (%)	45 (51.7)	233 (51.1)	0.92
Gestational age, mean±SD, week	38.5±1.1	38.8±1.1	0.10
Birth weight, mean±SD, g	3,026±380	3,138±349	0.007
Birth weight < 2,500 g, n (%)	5 (5.7)	9 (2.0)	0.06
Birth weight>3,800 g, n (%)	1 (1.1)	15 (3.3)	0.49
Percentage of weight loss from birth within the admission, mean±SD, %	5.8±2.4	5.5±2.3	0.37

Table 2: Univariate analysis of risk factors for neonatal hyperbilirubinemia

Factors	**NH n (%)**	**Non NH n (%)**	**Odds ratio (95% CI)**	**p-value**
Delivery method				
-Normal delivery	72 (82.8)	396 (86.8)	reference group	
-Vacuum extraction	11 (12.6)	18 (3.9)	3.4 (1.5-7.4)	0.003
-Forceps extraction	2 (2.3)	3 (0.7)	3.7 (0.6-22.3)	0.16
-Breech extraction	1 (1.1)	2 (0.4)	2.8 (0.2-30.7)	0.41
-Caesarian section	1 (1.1)	37 (8.1)	0.15 (0.02-1.10)	0.06
Cephalohematoma				
-Presence	2 (2.3)	4 (0.9)	2.7 (0.5-14.7)	0.25
-Absence	85 (97.7)	452 (99.1)	reference group	
Type of feeding				
-Exclusive breast feeding	25 (28.7)	146 (32.0)	0.9 (0.5-1.4)	0.55
-Breast and supplementary feeding	62 (71.3)	310 (68.0)	reference group	
ABO blood group				
- Mother O/infant A or B	19 (21.8)	53 (11.6)	2.1 (1.2-3.7)	0.02
- Other	66 (75.9)	382 (83.8)	reference group	
-Unknown	2 (2.3)	21 (4.6)		
G6PD enzyme status				
-Normal	58 (66.7)	397 (87.3)	reference group	
-Intermediate deficiency	11 (12.6)	26 (5.7)	2.9 (1.4-6.2)	0.006
-Complete deficiency	18 (20.7)	32 (7.0)	3.9 (2.0-7.3)	<0.001
Percentage of weight loss				
-> 10% weight loss	3 (3.4)	12 (2.6)	1.3 (0.4-4.7)	0.69
-≤ 10% weight loss	83 (95.4)	432 (94.7)	reference group	
-Unknown	1 (1.1)	12 (2.6)		
Alpha-thalassemia status				
-Non α^0-thalassemia carrier	82 (94.3)	425 (93.2)	reference group	
-α^0-thalassemia carrier	4 (4.6)	23 (5.0)	0.9 (0.3-2.7)	0.90
-Hemoglobin H disease	1 (1.1)	8 (1.8)	0.7 (0.1-5.3)	0.65

Table 3: Multivariate analysis of risk factors for neonatal hyperbilirubinemia

Factors	Adjusted odds ratio (95% CI)	p-value
Delivery method		
-Normal delivery	reference group	
-Vacuum extraction	4.2 (1.9-9.7)	0.001
-Forceps extraction	4.1 (0.6-27.2)	0.14
-Breech extraction	2.6 (0.2-33.0)	0.45
-Caesarian section	0.15 (0.02-1.1)	0.06
ABO blood group		
- Mother O/infant A or B	2.1 (1.1-3.9)	0.02
- Other	reference group	
G6PD enzyme status		
-Normal	reference group	
-Intermediate deficiency	3.4 (1.5-7.6)	0.004
-Complete deficiency	4.2 (2.1-8.2)	<0.001

Table 4: *UGT1A1* polymorphisms in G6PD deficient newborns classified by the presence or absence of neonatal hyperbilirubinemia

***UGT1A1* polymorphisms**	**NH**	**Non NH**	**Total**	**p-value**
Nucleotide 211 (G>A)				
-G/G	20 (71.4)	40 (71.4)	60 (71.4)	0.77
-G/A	8 (28.6)	15 (26.8)	23 (27.4)	
-A/A	0	1 (1.8)	1 (1.2)	
TATA promoter (TA7)				
-TA6/6	22 (75.9)	37 (66.1)	59 (69.4)	0.55
-TA6/7	7 (24.1)	18 (32.1)	25 (29.4)	
-TA7/7	0	1 (1.8)	1 (1.2)	

ภาคผนวก 3

Manuscript

Charoenkwan P, Tantiprabha W, Sirichotiyakul S, Taweephol R, Phusua A, Sanguansermsri T. Prevalence and molecular characterization of glucose-6-phosphate dehydrogenase deficiency in northern Thailand.

Prevalence and molecular characterization of glucose-6-phosphate dehydrogenase deficiency in northern Thailand

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Running title: G6PD mutations in northern Thailand

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Abstract

Neonatal cord blood screening for G6PD deficiency was conducted in 566 northern Thai newborns. Ninety (16%) had G6PD deficiency. The prevalence in male newborns was 17% (48 of 289 male newborns). The prevalence of female newborns having an intermediate deficiency and a complete deficiency of G6PD enzyme was 13% (37 of 277 female newborns) and 1.8% (5 of 277 female newborns) respectively. Six common G6PD variants previously reported in Thailand; G6PD Viangchan (871G>A), G6PD Mahidol (487G>A), G6PD Kaiping (1388G>A), G6PD Canton (1376G>T), G6PD Union (1360C>T) and G6PD Chinese-5 (1024C>T) were tested using polymerase chain reaction-restriction fragment length analysis. From 95 *G6PD* alleles tested, G6PD Mahidol (19), G6PD Kaiping (17), G6PD Canton (15) and G6PD Viangchan (13) are the most common mutations. These four mutations combined comprise about 70% of G6PD mutations in the population. The higher proportion of G6PD Mahidol, G6PD Kaiping, G6PD Canton in our population as opposed to G6PD Viangchan being the commonest mutations in the other parts of Thailand suggests a significant genetic drift from China and Myanmar into the region and represents a genetic continuum from southern China and Myanmar to northern and central Thailand.

Abstract word count: 189

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common inherited enzymopathy.[1] As G6PD deficiency protects against malarial infection, the prevalence is high in endemic area of malaria including southeast-Asia and Thailand.[1, 2] G6PD enzyme catalyzes a production of nicotinamide adenine dinucleotide phosphate (NADPH) in pentose phosphate pathway. NADPH is essential for the conversion of glutathione to a reduced form, which functions as an antioxidant. G6PD-deficient red blood cells are subjected to oxidation injury and premature hemolysis.[3]

Individuals with G6PD deficiency are usually asymptomatic, but may present with acute hemolysis when exposed to oxidizing agents. They are also at increased risk of neonatal hyperbilirubinemia. A small subset of G6PD-deficiency presents with chronic hemolysis.[3]

G6PD deficiency has an X-linked recessive inheritance. Symptomatic patients are mostly hemizygous males, and also the less common homozygous females. Some heterozygous females may have a decreased level of G6PD enzyme in the intermediate-deficient range, a phenomenon attributed to the skewed inactivation of X-chromosome as described by Lyon in 1961.[4] G6PD-deficient heterozygous females may experience acute hemolysis and are also at increased risk for neonatal hyperbilirubinemia.[5-7]

G6PD deficiency is common in Thai population with the prevalence rage of 3-18% in males.[8] G6PD Viangchan (871G>A) is reportedly the most common variant in the population in the central and southern Thailand.[9, 10] G6PD Mahidol (487G>A) was also reported to be the most common variant in the south.[11, 12] Other common mutations include G6PD Kaiping (1388G>A), G6PD Canton (1376G>T), G6PD Union (1360C>T) and G6PD Chinese-5 (1024C>T).(9, 10) The molecular features are similar to those in Laotians[13], Burmese[14, 15], Cambodians[16, 17], Malaysians[18, 19], southern Vietnamese[20], and southern Chinese[21, 22]. The finding suggests common ancestral origin of Thais with other population in southeast-Asia.

Northern Thailand is bordered by Myanmar and Laos, and is close to the southern part of China. This study took place in Chiang Mai, the major province of northern Thailand. The population comprises mainly Thais and assimilated Chinese. In the mountainous areas around Chiang Mai, there are several tribal groups including Karen, Lahu, Hmong, Lisu, Akha, Yao, Lua, Palong and Tai.

Herein we report the prevalence of G6PD deficiency and molecular characterization of the G6PD variants in northern Thai newborns. The study shows that G6PD deficiency has a high prevalence of 17% among male newborns. The mutations are heterogeneous. G6PD Mahidol, G6PD Kaiping, G6PD Canton, and G6PD Viangchan are the most common mutations.

Materials and Methods

Neonatal cord blood screening for G6PD deficiency was conducted. The study protocol was approved by the Institutional Ethics Committee. Informed consent was obtained from the mothers. Full-term newborns (gestational age 37-42 weeks) of mothers with uneventful pregnancy who were born at Chiang Mai University Hospital were included. After delivery of the newborn, umbilical cord blood was collected; 7 mL in ACD for G6PD assay and 5 mL in EDTA for DNA study. The blood samples were kept at 4^{o}C until analysis. The samples were tested for G6PD enzyme level. The samples which were G6PD deficient were further tested for G6PD mutations.

G6PD assay was performed according to the WHO method within 7 days of blood collection.[23] Average G6PD level in cord blood from male newborns was 12.5±2.3 IU/g Hb. Complete and intermediate deficiency of G6PD enzyme were defined by a level of less than 1.5 and 1.5-8.0 IU/g Hb respectively.

Genomic DNA was extracted from leukocytes by Chelex method.[24] Six common G6PD mutations previously reported in Thailand were tested. G6PD Viangchan (871G>A), G6PD Mahidol (487G>A), G6PD Kaiping (1388G>A), G6PD Canton (1376G>T), G6PD Union (1360C>T) and G6PD Chinese-5 (1024C>T) were tested using polymerase chain reaction-restriction fragment length analysis as previously described.[10, 25]

Results

Ninety of five hundred and sixty-six newborns (16%) had G6PD deficiency. The prevalence in male newborns was 17% (48 of 289 male newborns). The prevalence of female newborns having an intermediate deficiency and a complete deficiency of G6PD enzyme was 13% (37 of 277 female newborns) and 1.8% (5 of 277 female newborns) respectively.

The type of G6PD mutations are shown in table 1. G6PD Mahidol, G6PD Kaiping, G6PD Canton and G6PD Viangchan are the most common mutations. These four mutations combined comprise about 70% of G6PD mutations in the population.

Discussion

The prevalence of G6PD deficiency is high in northern Thai population. Interestingly, as high as 1.8% of female newborns had deficiency of G6PD enzyme in the homozygous range. The finding is comparable with the calculated probability from the allele frequency of G6PD variants (q) by Hardy-Weinberg principle. The allele frequency in our population is 0.1661, portion of G6PD deficient homozygous female equals to q^2, therefore the calculated prevalence is ~2.8% in females.

In northern Thai newborns, G6PD Mahidol, G6PD Kaiping, G6PD Canton and G6PD Viangchan are the most common mutations. G6PD Mahidol is the most common mutation, although the prevalence of these four mutations are about the same (range 14-20%). This finding is similar to the studies in southern Thailand.[11, 12] However, it is different from recent studies which reported G6PD Viangchan as the most common G6PD variant, in central Thai population by Nuchprayoon et al(10) and southern Thai population by Laosombat et al.[9]

G6PD Mahidol is the most common variant in Myanmar.[14, 15] The geographic proximity between Chiang Mai and Myanmar may explain the genetic similarity between the study population and the Burmese.

G6PD Kaiping and G6PD Canton are common in southern China and Taiwan.[21, 22, 26] These two mutations are prominent in the study population, supporting the assimilation of the Chinese to northern Thailand.

The presence of G6PD Mahidol, G6PD Kaiping, G6PD Canton and G6PD Viangchan point toward a common ancestry of the northern Thais with other southeast-Asian and southern Chinese population. The higher proportion of G6PD Mahidol, G6PD Kaiping, G6PD Canton in our population as opposed to G6PD Viangchan being the commonest mutations in the other parts of Thailand suggests a significant genetic drift from China and Myanmar into the region.

This study confirms the high prevalence of G6PD deficiency, and describes the new findings on characteristics of *G6PD* mutations in northern Thailand. The G6PD variants are heterogeneous and share similarity with surrounding regions, especially with Myanmar and Southern China.

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Table 1 G6PD mutations in northern Thais

Level of G6PD deficiency	Number of cases	Number of alleles	G6PD mutations						
			Mahidol	Kaiping	Canton	Viangchan	Union	Chinese-5	Unknown
Complete-deficient males	48	48	10	10	6	5	5	2	10
Intermediate-deficient females	37	37	9	6	6	4	2	0	10
Complete-deficient females	5	10	-	1	3	4	-	-	2
Total, n (%)	90	95 (100)	19 (20)	17 (18)	15 (16)	13 (14)	7 (7)	2 (2)	22 (23)

ภาคผนวก 4

บทความสำหรับการเผยแพร่

ผลการวิจัย

โครงการ ภาวะตัวเหลืองในทารกแรกเกิดในภาคเหนือของประเทศไทย:
บทบาทของภาวะพร่องเอนไซม์ จี-ซิก-พีดี และอัลฟ่า-ธาลัสซีเมีย

สนับสนุนโดย สำนักงานคณะกรรมการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

ภาวะตัวเหลืองในทารกแรกเกิดเป็นปัญหาที่พบได้บ่อยในทารกไทย ภาวะนี้มีความสำคัญเนื่องจากอาจก่อให้เกิดผลข้างเคียงที่รุนแรงได้แก่ ภาวะพิษของบิลิรูบินหรือสารสีเหลืองต่อเซลล์ประสาท ทำให้เกิดอาการทางระบบประสาทได้หลากหลาย ตั้งแต่ พฤติกรรมเปลี่ยนแปลง ชัก พัฒนาการช้า และความผิดปกติของการควบคุมการเคลื่อนไหว หรือในรายที่อาการรุนแรงอาจทำให้เสียชีวิตได้ ภาวะพิษของบิลิรูบินเป็นภาวะที่ป้องกันได้โดยให้การวินิจฉัยภาวะตัวเหลืองตั้งแต่แรกเริ่ม สืบหาปัจจัยเสี่ยงและให้การแก้ไขหรือรักษาประคับประคอง และให้การรักษาด้วยการส่องไฟ หรือในรายที่รุนแรงด้วยการเปลี่ยนถ่ายเลือดตามข้อบ่งชี้

อุบัติการณ์ของภาวะตัวเหลืองในทารกแรกเกิดจะแตกต่างกันตามเชื้อชาติ โดยพบในทารกชาวเอเชียมากที่สุด ตามด้วยทารกชนผิวขาว และทารกชนผิวดำ ตามลำดับ จึงเป็นที่น่าสนใจว่าทารกชาวเอเชียมีปัจจัยใดที่ส่งเสริมการเกิดภาวะตัวเหลืองดังกล่าว ปัจจัยที่อาจมีส่วนเกี่ยวข้อง แบ่งออกได้เป็น ปัจจัยที่ทำให้เกิดการทำลายเม็ดเลือดแดงและการสังเคราะห์บิลิรูบินเพิ่มขึ้น และความผิดปกติของกระบวนการเปลี่ยนรูปหรือการขับบิลิรูบิน ผู้วิจัยจึงได้ทำการศึกษานี้ โดยเน้นที่บทบาทของภาวะพร่องเอนไซม์ จี-ซิก-พีดี และอัลฟ่า-ธาลัสซีเมีย ซึ่งเป็นภาวะที่พบบ่อยในคนไทย และศึกษาเพิ่มเติมเกี่ยวกับยีนยูจีทีวันเอวันซึ่งเกี่ยวกับการขับบิลิรูบิน ในภาวะตัวเหลือง

จากการศึกษาพบว่า ทารกไทยในภาคเหนือมีอุบัติการณ์ของภาวะตัวเหลืองที่ต้องรับการส่องไฟสูงถึงร้อยละ 16 ปัจจัยเสี่ยงที่สำคัญของภาวะตัวเหลืองได้แก่ ภาวะพร่องเอนไซม์ จี-ซิก-พีดี ภาวะที่หมู่เลือดของแม่และลูกไม่เข้ากัน การคลอดด้วยเครื่องดูดสุญญากาศ ส่วนภาวะอัลฟ่า-ธาลัสซีเมียไม่เกี่ยวข้องกับการเกิดตัวเหลือง และความผิดปกติของยีนยูจีทีวันเอวันไม่ทำให้เกิดตัวเหลืองมากขึ้นในทารกที่มีภาวะพร่องเอนไซม์จี-ซิก-พีดี

การศึกษาเกี่ยวกับธาลัสซีเมียในทารกแรกเกิดกลุ่มเดียวกันนี้ พบว่า การตรวจนับเม็ดเลือดและวัดขนาดของเม็ดเลือดแดงสามารถใช้คัดกรองพาหะและโรคกลุ่มอัลฟ่า-ธาลัสซีเมียและฮีโมโกลบินผิดปกติบางชนิดได้ และการวิเคราะห์ฮีโมโกลบินด้วยวิธีไอโซอิเลคทริคโฟกัสซิ่งสามารถใช้วินิจฉัยโรคธาลัสซีเมียในทารกแรกเกิดได้

นอกจากนี้ จากการศึกษานี้พบว่า ความชุกของภาวะพร่องเอนไซม์ จี-ซิก-พีดีในทารกแรกเกิดไทยในภาคเหนือสูงถึงร้อยละ 17 ในเพศชาย และความผิดปกติทางพันธุกรรมที่ก่อให้เกิดภาวะนี้มีความหลากหลาย และแตกต่างจากที่พบในทารกไทยในภาคกลาง

ข้อมูลเหล่านี้จะเป็นหลักฐานสำคัญทางการแพทย์ เพื่อใช้อ้างอิงประกอบการพิจารณากำหนดแนวทางในการวางแผนการดูแลรักษาทารกแรกเกิดที่มีภาวะตัวเหลืองในประเทศไทย และทารกชาวเอเชียที่มีพื้นฐานทางพันธุกรรมที่คล้ายคลึงกัน รวมถึงแนวทางการตรวจคัดกรองภาวะอัลฟ่า-ธาลัสซีเมียและฮีโมโกลบินผิดปกติ และภาวะพร่องเอนไซม์จี-ซิก-พีดีได้ต่อไป