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SHORT COMMUNICATION

Haemoglobin Constant Spring (HbA2: c.427T>C) and Haemoglobin Adana (HbA2: c.179G>A) in jaundiced Malaysian term neonates with clinically significant hyperbilirubinemia

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Abstract

Introduction: Haemoglobin Constant Spring (Hb CoSp) and Haemoglobin Adana (Hb Adana), are two non-deletion type of α-thalassemia reported in Malaysia. Owing to their structural instability, they cause hemolysis and hyperbilirubinemia. This observational study was part of a large study investigating multiple factors associated with severe neonatal jaundice. In this part we aimed to determine the prevalence of Hb CoSp and Hb Adana and their association with clinically significant neonatal hyperbilirubinemia (SigNH, total serum bilirubin (TSB>290μmol/L)) among jaundiced Malaysian term neonates. *Materials and Methods:* The inclusion criteria were normal term-gestation neonates admitted consecutively for phototherapy. PCR-restriction fragment length polymorphism method was applied on DNA extracted from dry blood spot specimens of each neonate to detect for Hb CoSp and Hb Adana gene. Positive samples were verified by gene sequencing. *Results:* Of the 1121 neonates recruited (719 SigNH and 402 no-SigNH), heterozygous Hb CoSp gene was detected in only two (0.27%) neonates. Both were SigNH neonates (0.3% or 2/719). No neonate had Hb Adana variant. *Conclusion:* Hb CoSp was not common but could be a risk factor associated with SigNH. No Hb Adana was detected.

Keywords: Non-deletion alpha thalassemia, Haemoglobin Constant Spring gene, Haemoglobin Adana gene, significant neonatal hyperbilirubinemia

INTRODUCTION

Hyperbilirubinemia is the most common condition among normal term neonates which requires close evaluation and early treatment to prevent kernicterus spectrum disorders.[1,2] In 2014, 330,340 (64.6% of 511,865 annual livebirths) cases of neonatal jaundice were reported in Malaysia, and 58,580 of them required admission for treatment of hyperbilirubinemia.[3] Based on currently available laboratory tests in Malaysia, only 60% of the jaundiced neonates had a cause identified;[4] these were G6PD deficiency, fetal-maternal blood group incompatibility, sepsis and dehydration due to breast under-feeding.[3,4]

Alpha thalassemia (α-thalassemia) is a genetic disorder of haemoglobin synthesis due to either deletion of the α genes with reduced production of α-globin chains or non-deletion point mutation with resultant structural abnormalities of α-globin chains. Given their structural instability of the non-deletion type of α-globin, hemolysis and hyperbilirubinemia were observed to be more common than deletion type.[5,6] In Malaysia, α-Thalassemia is a common condition.[7-10] A large sample-sized study reported 80% of α-thalassemia in Malaysia were of the deletion type and 20% were non-deletion type.[7-10] The two most commonly reported non-deletion types of α-thalassemia in Malaysia were Haemoglobin Constant Spring (Hb CoSp)[7-11] and Haemoglobin Adana (Hb Adana).[7,8,12-14] At birth, fetal haemoglobin (Hb $F^{\alpha 2\gamma 2}$) is the predominant circulating haemoglobin. Affected newborns can thus be symptomatic at birth.[15] Universal newborn screening for α-thalassemia is currently not available in Malaysia due to cost

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and technical challenges. It is still uncertain whether it is a common cause of severe neonatal hyperbilirubinemia (total serum bilirubin (TSB) ≥342μmol/L) in this country. We, therefore, conducted a study with the aim to determine the prevalence of these two non-deletion types of α-thalassemia in jaundiced neonates and their association with clinically significant neonatal hyperbilirubinemia (SigNH, TSB >290μmol/L) in Malaysia.

MATERIALS AND METHODS

This was an observational study carried out in the Selayang Hospital (August 2014 through March 2016) on all term neonates (≥37 weeks' gestation) admitted for treatment of jaundice. The exclusion criteria were neonates who were premature, unwell, with major congenital malformations or no parental consent to participate. The main findings of this study have already been published recently[16] except for the findings on α-thalassemia. The Medical Research and Ethics Committee of the Ministry of Health of Malaysia (approval number NMRR-14-225-19651), and the Universiti Tunku Abdul Rahman Research Committee (U/SERC/10/2014) approved this study. Parents gave written consent for their neonates to participate in this study.

Upon admission, TSB was measured and a specimen of blood was obtained from each neonate for dry blood spots (DBS) onto Whatman FTA (Flinders Technology Associates) papers for molecular tests.

Detection of presence of Hb CoSp and Hb Adana gene

Ten punches of 1.2 mm spots from each neonate's DBS specimens were subjected to deoxyribonucleic acid (DNA) extraction using a standard protocol (BiolineInc, U.S.A). The PCR-restriction fragment length polymorphism (RFLP) method was applied to detect for the known Hb CoSp and Hb Adana variant sites in the α-thalassemia gene reported in Malaysia previously.[13,17] The natural or mutagenic primers, restriction enzymes, and digested restriction fragment sizes of these two variants are listed in Table 1. The PCR mixture (25 μl) consisted of 200 ng of DNA, 1 μl of each primer (20 μM each), 12.5 μl of Mytaq Mix (BiolineInc, U.S.A) and 8.5 μl of water (ddH_2O). The PCR amplification was performed in a DNA thermal cycler (Applied Biosystems, Veriti, U.S.A.) for 35 cycles of initial denaturation for 5 min at 95°C, annealing for 15 seconds at 62-66°C, primer extension for 90 sec at 72°C. The PCR product was digested with the appropriate restriction enzyme and analyzed on 3% agarose gel (NHK Bioscience Solutions Sendirian Berhad, Malaysia). For confirmation of positive results detected by PCR-RFLP method, gene-sequencing was carried out on the respective PCR products using Applied Biosystems 3730XL DNA Analyzer and Applied Biosystems Sequence Scanner Software for sequence result analysis. Sequence data were compared with the GenBank DNA Database using BLASTn searches to determine the alignment (% identity) between primers and sequences containing mutations using the National Centre of Biotechnology Information (NCBI) BLAST network server available from http://www.ncbi.nlm.nih.gov/. All the results matched with those determined by the sequencing method.

Statistical analysis

SPSS 13.0 for Windows software program (SPSS Inc., Chicago, IL, U.S.A.) was used for statistical analysis.

RESULTS

Of the 1121 jaundiced neonates recruited, 51.0% (n=572) were males, 74% (n=830) were Malays, 16.2% (n=182) Chinese, 2.9% (n=33)

TABLE 1: Natural or mutagenesis primers, restriction enzymes, and Haemoglobin Constant Spring and Haemoglobin Adana gene variations[13,17]

Position (cDNA)	Primers	Sequence	Restriction enzyme	Result (bp)
Hb CS	Hb CS-F	5′GCG GT TGC GGG AGG T 3′	*Taq*$^{\alpha}$-*I*	T 222
(427T>C)	Hb CS-R	5′GAA CGG CTA CCG AGG CTC CAG CTC3′		C 200+22
Hb Adana	Hb Adana-F	5′GCT CTG CCC AGG TTA AGG GCC TCG3′	*Taq*$^{\alpha}$-*I*	G 285+175
(179G>A)	Hb Adana-R	5′GGG AGG CCC ATC GGG CAG GAG GAA C 3′		A 285+153+43

Note: Hb CS = Haemoglobin Constant Spring; Hb Adana= Haemoglobin Adana; bp= base pairs

Indians, and 6.8% (n=76) other ethnic groups. Their mean birth weight was 3065g (±437), and mean gestational age was 38.5 weeks (±1.0). There were 719 (64.1%) neonates with SigNH (median peak TSB = 326 μmol/L [interquartile range (IQR): 307, 350], range 291-479 μmol/L); and 402 (35.9%) with no-SigNH. (median peak TSB = 261 μmol/L [IQR: 236, 285], range 155-290 μmol/L).

Heterozygous mutation in Hb CoSp (Fig. 1) was detected in two of the 1121 jaundiced neonates screened, giving a prevalence of 0.18%. Both neonates had SigNH, giving a prevalence of Hb CoSp of 0.3% (2/719) in SigNH. Both were females, giving a prevalence rate of 0.57% (2/348) among females with SigNH, and a prevalence of 0.36% (2/549) among all jaundiced females. One was a Malay (peak TSB of 376 μmol/L) and the other was a Chinese (peak TSB of 299 μmol/L) neonates. The prevalence of Hb CoSp in jaundiced Malays at 0.12% (1/830) was not significantly different from the prevalence in Chinese at 0.55% (1/182) (p values=0.327). No neonates were detected to have Hb Adana.

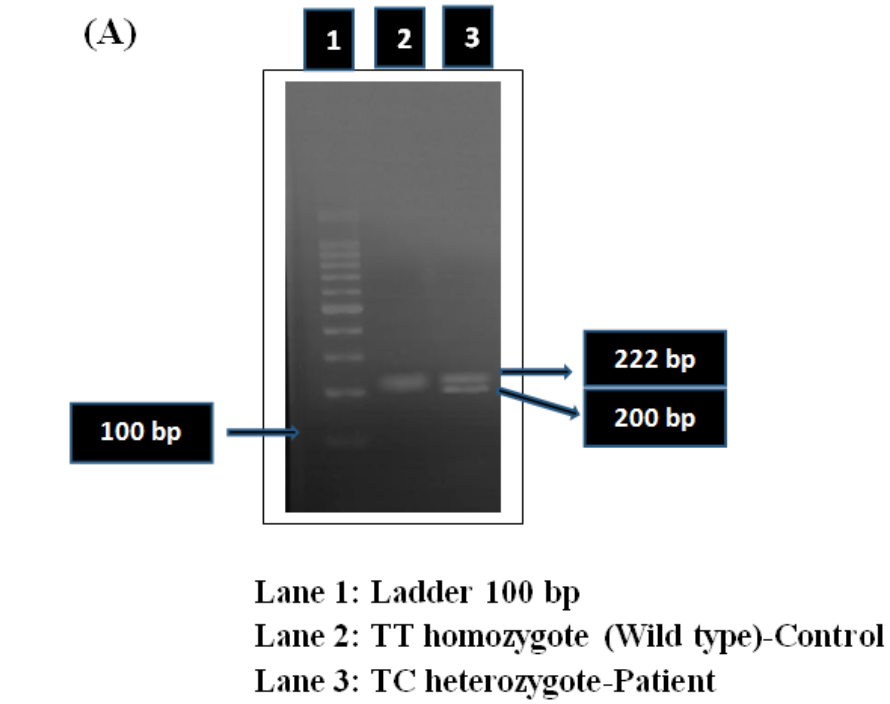

FIG. 1: (A) PCR and restriction pattern of *Hb CS* (427T>C) gene. Lane 1 is a 100 bp DNA marker. Lane 2 and 3 are restriction fragment pattern of PCR products after digesting with *Taq*$^{\alpha}$*I* and running in 3% agarose gel. (B) Sequence chromatogram of *Hb CS* (427T>C) gene showing TC heterozygous mutation of one of the patients.

DISCUSSION

Our study showed that the prevalence of Hb CoSp in all jaundiced neonates admitted (at 0.18%) was much lower than those reported in large sample studies of adults with microcytosis (n=5016)[7], adolescents with abnormal levels of HbA_2 or HbF (n=8366)[8], and pregnant women with microcytosis (n=650).[9] In these studies, the prevalence of Hb CoSp was reported to be 3.2%[7], 0.23%[8], and 1.4%[9], respectively. One most likely explanation could be that we screened all neonates rather than those with microcytosis or abnormal haemoglobin based on electrophoresis.[7-9] Nevertheless, in the present study, Hb CoSp was detected only in jaundiced neonates with SigNH, suggesting that Hb CoSp may be a potential risk factor associated with SigNH.

Hb Adana was reported to be the second commonest non-deletion α-thalassemia in the large sample study (n=5016) of high-risk patients by Rahimah *et al*.[7] In our study, Hb Adana (c.179G>A) was not detected in any of the 1121 jaundiced neonates, suggesting that Hb Adana is very rare in the general population.

The strength of our study is the large number of neonates recruited and all positive results were confirmed by gene sequencing. In view of the fact that there is still a large proportion of jaundiced neonates with severe hyperbilirubinemia of unknown cause in Malaysia[3,4], and that adult studies showed that deletion type of α-thalassemia was more common[7-9], molecular studies should be carried out in neonates to determine whether this is a significant risk factors associated with severe hyperbilirubinemia in Malaysia. This will help us to determine whether universal newborn screening for α-thalassemia should be carried out in our country to identify neonates at risk of developing SigNH due to α-Thalassemia as practised elsewhere.[18]

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Conflict of interest: The authors declare they have no conflict of interest.

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