EVALUATION OF MOLECULAR MARKERS ASSOCIATED WITH SIGNIFICANT NEONATAL HYPERBILIRUBINEMIA OF THE THREE ETHNIC GROUPS IN MALAYSIA

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JANUARY 2020

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By

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A thesis submitted to the
Faculty of Medicine and Health Sciences,
Universiti Tunku Abdul Rahman,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Medical Sciences
January 2020

To my beloved family and husband

ABSTRACT

EVALUATION OF MOLECULAR MARKERS ASSOCIATED WITH SIGNIFICANT NEONATAL HYPERBILIRUBINEMIA OF THE THREE ETHNIC GROUPS IN MALAYSIA

Shwe Sin

The aim of this study was to identify the major genetic risk factors associated with significant hyperbilirubinemia among Malaysian jaundiced newborn infants and also the prevalences of genetic variants of these four genes. Significant neonatal hyperbilirubinemia (SigNH) was defined as total serum bilirubin (TSB) leveled off at 17 mg/dL (≥291 μmol/L) in the term infants. In addition, this study determined the association between co-expression of two or more of genetic variants with significant hyperbilirubinemia in Malaysian newborn infants. This study identified the genetic variants of UDP-glucuronosyltransferase (*UGT*) 1A1, glucose-6-phosphate dehydrogenase (*G6PD*), solute carrier organic anion transporter family membrane 1B1 (*SLCO1B1)* and non-deletional alpha-

thalassemia in Malaysia newborn infants with and without significant hyperbilirubinemia by using PCR-RFLP (Polymerase Chain Reaction- Restriction Fragment Length Polymorphism) method. Samples which were positive for the detection of mutations according to the above described methods were verified by DNA sequencing.

This study recruited 1121 hyperbilirubinemic neonates. The study indicates that the percentage of significant neonatal hyperbilirubinemia was 62.1% (696 of 1121) in Malaysian neonates. Males had 51.2% (574 of 1121) and females showed 48.8% (547 of 1121) in this study. In addition, Malay had the highest percentage of hyperbilirubinemia (74.9%) while Indian had lowest number in our study population (3.2%). Malaysian Chinese and others showed 16.9% and 5.0% respectively. There were no significant differences in the gender and ethnic distribution between SigNH and non-SigNH in the study population.

The result of the present study showed that 70.7% (793/1121) of the main ethnic groups of neonates carried a *UGT1A1* gene mutation. This study detected *UGT1A1* gene variants 211G>A (3.2%), 686C>A (1.1%), 1091C>T (0.4%), 1456T>G (1.3%), c.-3279T>G (76.2%) and promoter A$(TA)_n$TAA (17.9%). The commonest mutations found were c.-3279T>G and TA repeat at promoter region. Moreover, genotypic and allelic distribution of variants *UGT1A1* gene 211G>A and TA promoter region repeat were significantly associated in their frequencies with SigNH group showed OR: 6.26 ($p = 0.02$) and OR: 3.26 ($p = 0.001$). However, the

c.-3279T>G variant mutation was significantly associated in the frequency with non-SigNH showed OR: 0.31 (p = 0.001). In addition, all randomly selected 300 samples of SigNH (n = 200) and non-SigNH (n = 100) showed homozygous 214G>C mutation in sequencing analysis.

Out of total 1121 subjects studied, 3.1% (35/1121) were found to have *G6PD* mutation. This study detected variant mutations of *G6PD* nt 1388G>A (60.0%), nt 871G>A (17.1%), nt 487G>A (14.3%), nt 1376G>T (5.7%) and nt 1003G>A (2.9%). The predominant variation in the *G6PD* gene was nt 1388 G>A (*G6PD* Kaiping), followed by 871 G>A (*G6PD* Viangchan). None of the *G6PD* gene mutations showed significant difference in their frequencies in SigNH and non-SigNH groups. The present study did not find any mutations of nucleotide 563 C>T, 383T>C, and 131 C>G in our study population.

In this study, 49.3% (553/1121) were found to have *SLCO1B1* gene mutation. The study observed the variants c.388G>A mutation (53.3%) and c.521C>T mutation (46.7%). Logistic regression analysis of the variant 388G>A mutation showed significance risk for SigNH group (p = 0.05).

There were two cases of heterozygous variation of non-deletional alpha-thalassemia Hb CS gene detected in SigNH group (0.2%), one Malaysian Malay and one Malaysian Chinese. No Hb Adana gene mutation was found either in SigNH or in non-SigNH cases.

A total of 357 coexpressed cases were found in both SigNH and non-SigNH. From these, 97.5% (348 of 357) showed 2 variants coexpression and 2.5% (9 of 357) showed 3 variants coexpression.

In conclusion, the recent study can show the mutation patterns and risks associated with significant neonatal hyperbilirubinemia in the three ethnic groups of Malaysia. Out of 18 variants studied, 14 variants mutations of *UGT1A1*, *G6PD*, *SLCO1B1* and α -thalassemia genes were detected. From these, promoter A(TA)nTAA, variant 211G>A of *UGT1A1* gene and *SLCO1B1* 388G>A variants showed significance risk for SigNH group. In addition, co-expression of *SLCO1B1* variant c.521T>C and *UGT1A1* variant c.-3279T>G mutations showed significant difference in the frequency in SigNH and non-SigNH groups ($p = 0.03$).

ACKNOWLEDGEMENT

I would like to express my gratitude to Emeritus Professor Dr. Cheong Soon Keng, Dean, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman and all members of the Research and Ethical Committee of Universiti Tunku Abdul Rahman, for their kind permission to conduct this study.

My deepest and sincere gratitude must go to my supervisor Professor Dr. Alan Ong Han Kiat, Deputy Dean, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, for his kind guidance, continuous close supervision in molecular field and sharing technical knowledge concerning molecular technology and invaluable advice for throughout this study.

I am deeply indepted and grateful to my co-supervisor Emeritus Professor Dr. Boo Nem Yun, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, for her kind guidance, continuous supervision, invaluable suggestions and opinion throughout my study.

My heartfelt thanks to Professor Dr. Yap Sook Fan, Deputy Dean for Postgraduate study, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, for her kind support and encouragement.

My sincere thanks also go to Professor Ts. Dr. Lim Yang Mooi, Head of Post-graduate Programme, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, for her kind suggestions, comments and invaluable supports.

My special thanks are conveyed to Associate Professor Dr. Leong Pooi Pooi, Head of Preclinical Science Department, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, for her kind suggestions, comments and invaluable supports

My great thanks also would like to extend to Dr. Chee Seok Chiong, Dr. Michelle Ling Min Min, Dr. Maslina Mohamed, Dr. Anita Kaur Ahluwalia, Department of Paediatrics, Salayang Hospital for the clinical demographic data collection and sample collection from the patients under their care.

My special thanks are conveyed to Dr. Mohammed Abdulrazzaq Jabbar and Dr. Gary Low for their kind help with statistical analysis of the data obtained.

I am very greatful to all patients who participated in this study. I also thank to Science Officers of the Postgraduate Laboratory, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman and all my Postgraduate colleagues for their kind help and unreserved supports throughout this research.

I would like to thank to all my senior and junior colleague staffs, Faculty of Medicine and Health Sciences, Universiti Tunku Abdul Rahman, for their kind support during the course of Ph.D.

My great thanks also would like to extend to Universiti Tunku Abdul Rahman as this study was fully funded by research grants, UTARRF 2015 (6200/B03) and UTARRF 2016 (6200/S59).

Last but not least, a big bouquet of thanks to my dearest family for their understanding and support when I was facing difficulties. Without my family I would not be who I am today and I am thankful for all that they have done for me.

APPROVAL SHEET

This thesis entitled "**EVALUATION OF MOLECULAR MARKERS ASSOCIATED WITH SIGNIFICANT NEONATAL HYPERBILIRUBINEMIA OF THE THREE ETHNIC GROUPS IN MALAYSIA"** was prepared by SHWE SIN and submitted as partial fulfillment of the requirements for the degree of Ph.D. of Medical Sciences at Universiti Tunku Abdul Rahman.

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SUBMISSION OF THESIS

It is hereby certified that **SHWE SIN** (ID No: **15UMD01263**) has completed this thesis entitled "**EVALUATION OF MOLECULAR MARKERS ASSOCIATED WITH SIGNIFICANT NEONATAL HYPERBILIRUBINEMIA OF THE THREE ETHNIC GROUPS IN MALAYSIA**" under the supervision of Professor Dr. Alan Ong Han Kiat (Supervisor) from the Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences, Emeritus Professor Dr. Boo Nem Yun (Co-Supervisor) from the Faculty of Medicine and Health Sciences.

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Yours truly,

_____

(SHWE SIN)

DECLARATION

I, SHWE SIN hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(SHWE SIN)

Date _____

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LIST OF ABBREVIATIONS

PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
P	Probability
ORs	Odd Ratios
Tm	Melting temperature
TSB	Total Serum Bilirubin
SigNH	Significant neonatal hyperbilirubinemia
Non-SigNH	Non-significant neonatal hyperbilirubinemia
TcB	Transcutaneous bilirubin
RBC	Red blood cells
UGT1A1	Uridine diphosphate glucuronosyltransferase 1A1
G6PD	Glucose-6-phosphate dehydrogenase
SLCO1B1	Solute carrier organic anion transporter family member 1B1
OATP	Organic anion transporter polypeptide protein
AAP	American Academy of Pediatric

CHAPTER 1

INTRODUCTION

Jaundice characteristically consequences from the accumulation of pigment of unconjugated bilirubin in the mucous membrane as well as in the skin. Neonatal jaundice is defined as a serum total bilirubin level >5 mg/dL (>86 umol/L) (Keren *et al*., 2009). It is common among infants, affecting up to nearly 50-60% of newborns in the first week of life (Ullah, 2016) with one in two infants globally affected (Brits, 2018). A pediatric disease or complex trait presents in severe neonatal jaundice, in which the total bilirubin is raised to ≥20 mg/dL (342 umol/L) (Bhutani et al., 2004 and Newman *et al*., 1999) and this severe hyperbilirubinemia condition is changed from significant neonatal hyperbilirubinemia which is total serum bilirubin ≥17 mg/dL (291 umol/L).

This disease may present during the neonatal period depending on the underlying etiology. Few patients have significant underlying disease even though nearly 60% of the term infants have clinical jaundice in the early neonatal period. Newborns without recognized risk conditions seldom have total serum bilirubin levels beyond 12 mg/dL (>205 umol/L) (Porter and Dennis, 2002). It has been described that the likelihood to develop markedly high bilirubin levels

also increases as the number of risk factors increases (Porter and Dennis, 2002).

Hyperbilirubinemia is the most frequent clinical condition among newborns, although it is a postnatal benign transitory occurrence of no obvious clinical importance. The serum total bilirubin (TSB) of affected neonates may increase to dangerous points in some cases and this causes a direct hazard of brain injury (Watchko, 2009, Greco, 2016). Furthermore, acute condition of bilirubin encephalopathy may occur, generally progressing into chronic bilirubin encephalopathy and lifelong neurodevelopmental impairment, termed kernicterus (Alken, 2019), a destructive, debilitating state characterised by the clinical conditions of athetoid dyskinetic cerebral palsy, increased occurrence of sensorineural hearing loss, vertical gaze palsy, and tooth enamel hypoplasia due to the consequence of toxicity of cells because of bilirubin effect (Watchk, 2006). Moreover, severe neonatal hyperbilirubinemia can also selectively induce temporal and occipital lobe seizures (Zhang, 2018). In addition, East Asian neonates have a greater incidence of hyperbilirubinemia as that of other ethnicities and a general increased risk for a total serum bilirubin levels of ≥342 umol/L (≥20 mg/dl) was commonly stated (Newman *et al*., 2000).

The basic biologic cause of hyperbilirubinemia is frequently multifactorial. The 2004 AAP (American Academy of Pediatric) guideline has been used for specific assessment, and management recommendation (Huang *et al*., 2004). The

main risk conditions for the severe jaundice in infants of 35 or more weeks gestation is shown in Table 1.1.

Table 1.1: Major risk factors for the development of severe neonatal hyperbilirubinemia in newborns of ≥35 weeks gestation (American Academy of Pediatric., 2004)

Major risk factors for the development of severe neonatal hyperbilirubinemia in newborns of ≥ 35 weeks gestation
≥35 weeks gestation
- Blood group incompatibility with positive direct Coombs test; other known hemolytic disease (eg, *G6PD* deficiency)
- East Asian ethnicity
- Exclusive breastfeeding, mainly if nursing is not going well and weight loss is extreme
Jaundice observed in the first day of life
- Previous sibling received phototherapy
- Cephalohematoma or obvious bruising
-Predischarge TSB or TcB level in the high risk zone

Even though each risk factor embraces the potential to be significant contributor to marked neonatal hyperbilirubinemia, for instance, exclusive breast feeding and blood group incompatibility, they are more often detected in combination with others. In newborns who had one significant risk factor had ORs: 8.46 ($p< 0.001$), ORs: 22.0 ($p< 0.001$) in two risk factors and ORs: 88.0 ($p<0.001$) in neonates with three risk factors, individually (Huang *et al*., 2004).

Furthermore, the effect of genetic variation is increasingly acknowledged (Watchko, 2010). The heritable conditions that may cause hemolysis and hyperbilirubinemia in neonates are uridine diphosphate glucuronosyltransferase 1A1 (*UGT1A1*), solute carrier organic anion transporter family member 1B1 (*SLCO1B1*), glucose-6-phosphate dehydrogenase (*G6PD*), and alpha thalassemia mutations (Watchko, 2012).

Genetic mutations on uridine diphosphate glucuronosyltransferase 1A1 (*UGT1A1*) have been revealed to be greatly associated with conditions of unconjugated hyperbilirubinemia, and *UGT1A1* is the main enzymatic protein for bilirubin conjugation (Servedio, 2005). The enzymatic protein actions reduced to 60% and 32% of normal in individuals with the *UGT1A1* gene G211A in the homozygous and heterozygous status respectively (Yamamoto, 1998). The homozygote genetic mutation in the promoter area of the *UGT1A1* in which (TA) dinucleotide is inserted in the TATA box alike sequence, forming $A(TA)_7TAA$ nucleotide sequence was shown in unconjugated hyperbilirubinemic patients mostly with Gilbert syndrome (Gupta, 2005). In addition, *UGT1A1* enzyme action was lowermost in hepatocytes from the homozygote (TA) promoter (7/7) and intermediary in the heterozygote (6/7), compared with the normal homozygote (6/6) (Raijmakers, 2000). Furthermore, the *UGT1A1* promoter transcriptional activity was reduced to 60% in the *UGT1A1* nt c.-3279T>G gene defect (Sugatani, 2002).

Previous studies on the relationship between neonatal jaundice and *UGT1A1* genetic polymorphism have been conducted, in which studies of Caucasian populations, including Sephardic Jews (Kaplan, 1997), Americans (Bancroft, 1998), Italians (Iolascon, 1998), and British (Monaghan, 1999) showed that *UGT1A1* genetic mutation is related with neonatal jaundice. Moreover, *UGT1A1* gene mutation was detected to be a risk condition for neonatal jaundice among case-control studies of Japan (Mauro, 1999), Taiwan (Huang, 2004) and India (Agrawal, 2009). Numerous studies of the TA promoter and nt G211A mutations in Southeast Asian population have been described (Yusoff, 2006), including Malaysia (Yusoff, 2010, Azlin, 2011, Teh, 2012,). However, the nt G211A variation of *UGT1A1* gene has been stated as the greatest common independent risk condition of neonatal hyperbilirubinemia in individual ethnic groups with small sample size that does not reflect the overall mutation pattern among the main ethnic group representative of Malaysian population (Kaplan, 1997).

Glucose-6-phosphate dehydrogenase, a main enzymatic protein, that involves the initial and the rate-limiting stage in the metabolic hexose monophosphate shunt path. Its main part in metabolism is to generate cytoplasmic reducing influence in the formation of reduced nicotinamide adenine dinucleotide phosphate. Complete appearance of the mutated gene is detected in the hemizygote male and the homozygote female because the human *G6PD* gene is situated on Xq28. A varied population of normal and cells with enzyme deficiency be able to be detected in the female heterozygote (Johnson, 2009). *G6PD* deficiency is

existing in >400 million people global, is the greatest common congenital enzymatic protein defect in males (Salvati, 1999). This condition ensues most often in certain populations of Mediterranean, African, and Asian. The worldwide distribution of this condition is strangely alike to that of malarial infection (Cappellin, 2008). A minimum of 202 mutations producing *G6PD* deficiency have been described in many peoples in the world (Doss, 2016). Deficiency of *G6PD* has been recognized as one of the most frequent reasons of severe neonatal hyperbilirubinemia in Malaysian people (Selvaraju, 1999) with a total incidence of around 3% among male gender and is more prevailing in Malay and Chinese ethnicity and less frequent among Indians (Singh, 1986, Hon, 1989). A small group of male *G6PD*-deficient Malaysian Chinese and subsequently in Malays *G6PD*-deficient individuals (Ainoon, 1995, Ainoon, 1999, Ainoon, 2003) were previously reported.

Solute carrier organic anion transporter protein family 1B1 (*SLCO1B1*) gene encodes the organic anion transporter polypeptide-2 protein (*OATP2*), otherwise known as *LST1* and *OATP-C*. It is a transporter uptake protein of human hepatocytes, situated on the basolateral membrane, which transported unconjugated bilirubin to the hepatocytes from the blood circulation (Wolkoff, 1987). Studies have described that *SLCO1B1* gene mutations of G388A and T521C may affect patients result in neonatal jaundice by affecting uptake of hepatic bilirubin (Watchko, 2010). A high prevalence of 388G>A and 521T>C variants were identified in Chinese populations (73.4% and 14.0%) (Xu, 2007). The meta-analysis

review results showed that *SLCO1B1* variant G388A is related with an increased threat of neonatal jaundice in Chinese populations, but not found in Whites, Latin American, Thai or Malaysian and *SLCO1B1* nt T521C variation revealed a reduce threat of neonatal jaundice in China, while no significant associations were detected in Whites, Brazil, Thai, Asia, and Malaysia (Liu, 2013). However, studies conducted in Malaysia showed that *SLCO1B1* nt 388G>A is the most common mutation in Malaysian Chinese (79.5%) and nt 521T>C is highest in Malaysian Indians (47.9%) (Wong, 2012).

Alpha thalassemia is a genetic disease of hemoglobin synthesis affecting 5% of world's population, and is characterised by a decline in the synthesis of alpha globin chains (Vichinsky, 2009). Although deficiencies in hemoglobin structure or synthesis occasionally noticeable in the newborn period, the α-thalassemia syndromes are the most possible to be clinically obvious in newborns. Over 30 structural variants of α-globin gene were described in the database of the human gene mutation. Non-deletional α^{+}-thalassemia mutations (e.g., Hb Adana and Hb constant spring) contribute to α-globin structural changes (Wee, 2005). A patient in a Chinese family with hemoglobin H disease in Constant Spring, Jamaica, has firstly been detected with Hb constant spring (Hb CS) (Milner, 1971). In Malaysia, the incidence of Hb CS has been reported in Malay (2.24%), Chinese (0.66%) and Indian (0.16%) adult populations correspondingly (Lie-Injo, 1977, Wee, 2005) and also was detected amongst Aborigines ("Orang Asli" people) in West and East Malaysia (Ganesan, 1975). Hb Adana remains a greatly unstable hemoglobin

resultant from the α1- or α2-globin gene mutation at codon 59 (HbA1: G179A or HbA2: G179A) (Cürük, 1993, Traeger-Synodinos, 1999). Hb Adana has been reported in an adult Malay male in Malaysia (George, 2009). However, Hb CS and Hb Adana have not been reported in Malaysian newborns with significant neonatal hyperbilirubinemia.

Impair hepatic bilirubin metabolism with heritable determined neonatal jaundice is caused by the genetic interactions among *UGT1A1*, *SLCO1B1*, and *G6PD* variant alleles with the significance of gene coupling polymorphisms (Stevenson *et al*., 2012). Coexpression case of *UGT1A1* with *G6PD*A^{-} mutation together with *SLCO1B1* variant was found more frequently in case groups of White and Chinese population (Watchko and Lin, 2010). Moreover, coexpression of ≥2 and ≥3 variant alleles polymorphisms were 15.7% and 4.6% respectively in case group (Watchko and Lin, 2010).

Although *UGT1A1*, *SLCO1B1*, *G6PD* and α-thalassemia genetic studies were carried out in Southeast Asia including Malaysia, these studies were done on small to moderate sample population and targeted a single ethnic group, so as the existing research data are not comprehensive. However, a bigger study population with inclusion of three main ethnic groups was conducted in our study and it is more realistic representation of Malaysian population. Moreover, *UGT1A1* gene at nucleotide 686C>A, nucleotide 1091C>T, and nucleotide 1456T>G and α-

thalassemia have not been examined among Malaysian hyperbilirubinemic neonates.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used in this study as this is an uncomplicated laboratory procedure applied to study the causes of genetic mutations and variations; this technique is principally valuable in studies of research in multifactor inherited diseases and it is a somewhat uncomplicated, inexpensive and precise method. A conserved DNA sequence region is amplified, followed by digestion with restriction endonuclease enzyme, which can show genetic variation between samples.

The benefits of PCR-RFLP are inexpensive and absence of requirement for advanced tools. It is also an extremely strong methodology with worthy exchangeability between laboratories. Furthermore, the PCR-RFLP analysis design generally is uncomplicated and can be accomplished using community available programs.

Problem Statement

Jaundice is the most frequent illness in the early neonatal period and it is the commonest reason of readmission during neonatal period (Swarnim, 2017). There are many factors causing jaundice in newborns, both physiological and pathological. If not treated early, complications associated with jaundice can cause

permanent neurological damage. Thus, Ministry of Health Malaysia has since the 1980's, taken various measures to address the issue of jaundice in newborns.

Neonatal jaundice is one of the important contributors to neonatal morbidity and mortality which has remained very high inMalaysia. National survey in Malaysia demonstrated that 75% of neonates contracted jaundice in the first week of life, and 25-30% of these cases developed severe neonatal jaundice (Wong, 2013). Various Malaysian healthcare facilities including the Malaysian Ministry of Health and private hospitals, have produced educational programme, neonatal jaundice is still very common problem in Malaysia (Boo, 2011).

Available evidence suggests that low- and middle -ncome countries brae the greatest burden of severe neonatal hyperbilirubinemia characterized by disproponately high rates of morbidity, mortality and neurodevelopmental disorders compared to high-income countries (Olusanya, 2015). Despite the intensive management and regular follow-up of newborn infants at home by the Maternal and Child Health nurses, many Malaysian infants are still developing significant hyperbilirubinemia and the number of infants admitted with severe hyperbilirubinemia of TSB >342 umol/L was still high (Status Report on Children's Rights in Malaysia by Child Right Coalition Malaysia, December 2012). Hence, Malaysian public hospitals are overcrowded with jaundiced neonates that causes economic and management burden. In addition, these hyperbilirubinemic newborns are still at high risk of brain damage.

Furthermore, studies on co-expression of different genes among neonatal hyperbilirubinemia in Malaysia is lacking. Numerous genetic factors have been recognized as a risk factors for emerging severe jaundice. As such by combining a TSB measurement with the molecular and clinical factors, a better predictive assessment on which newborns are at greater risk will benefit caretakers in birth management plans, hospitalization, and postdischarge outpatient follow-up and assessment (Maisles *et al*., 2009). This research will therefore further examine the relations between demographic factors and genetic contributions to neonatal jaundice. To the best of our information, this is the first evaluation and study of four genes; *UGT1A1*, *G6PD, SLCO1B1,* and α-thalassemia together with clinical demographic factors associated with significant neonatal hyperbilirubinemia in Malaysia.

RESEARCH QUESTIONS

Our research questions:

(1) Are certain genetic variants of *UGT1A1, G6PD, SLCO1B1* and non-deletional alpha-thalassemia either individually or collectively present at higher prevalence among the three ethnic groups of Malaysia?

(2) Do genetic variants of *UGT1A1, G6PD, SLCO1B1* and non-deletional alpha-thalassemia show any form of association with the prevalence of significant neonatal hyperbilirubinemia?

(3) Does the coupling of two or more genetic variants show higher association as compared to that of single gene variant in significant neonatal hyperbilirubinemia?

RESEARCH HYPOTHESIS

(1) Certain genetic variants of glucose-6-phosphate dehydrogenase (*G6PD*), *SLCO1B1*, *UGT1A1* and non deletional alpha-thalassemia are associated with significant neonatal hyperbilirubinemia.

(2) The coupling of two or more molecular variants is associated with significant neonatal hyperbilirubinemia compared to that of single gene variant.

GENERAL OBJECTIVE

- To determine whether the major demographic factors and molecular variants are significant predictors of significant hyperbilirubinemia in Malaysian newborn infants.

SPECIFIC OBJECTIVES of this study are as outlined below:

1. To determine the prevalence of significant hyperbilirubinemia among Malaysian jaundiced neonates in the study sample.
2. To compare the prevalence of common genetic variants of glucose-6-phosphate dehydrogenase (*G6PD*), UDP-glucuronosyltransferase 1A1 (*UGT1A1*), solute carrier organic anion transporter family member 1B1 (*SLCO1B1*) and non-deletional alpha-thalassemia in Malaysian newborn infants with and without significant hyperbilirubinemia.
3. To determine the association between co-expression of two or more of the above genetic variants with significant hyperbilirubinemia in Malaysian newborn infants.
4. To determine whether the molecular variants are significant predictors of significant hyperbilirubinemia in Malaysian newborn infants.

Thesis outline

Chapter 1 gave an introduction on neonatal hyperbilirubinemia and pathogenesis of neonatal jaundice was discussed. Problem statement, research questions, research hypothesis, general objective and specific objectives were described.

Chapter 2 discussed the literature review on neonatal hyperbilirubinemia, catabolism of heme, pathogenesis and specific genotypes associated with neonatal hyperbilirubinemia were discussed. This is followed by research methodology of genotype studies in Chapter 3. Chapter 4 to 7 addressed the results, discussions and conclusions of glucose-6-phosphate dehydrogenase (*G6PD*), UDP-glucuronosyltransferase 1A1 (*UGT1A1*), solute carrier organic anion transporter family member 1B1 (*SLCO1B1*) and non-deletional alpha-thalassemia gene polymorphisms and mutations respectively. Coexpression cases were described and discussed in Chapter 8. Lastly, Chapter 9 concluded the findings and discussed recommendations for future study.

CHAPTER 2

LITERATURE REVIEW

2.1 Neonatal Jaundice

Jaundice is well-defined as a yellow discoloration of the sclera, skin, and mucous membrane caused by increased circulating bilirubin levels >5 mg/dL (>86 umol/L). “Jaundice” means *yellow* which is derived from the French word *jaune*. Jaundice is a term used to describe the visible manifestation of elevated serum concentrations of bilirubin. The first step in the evaluation of jaundice is laboratory determination of fractionated bilirubin, as the diagnostic approach differs significantly for unconjugated and conjugated hyperbilirubinemia. In unconjugated hyperbilirubinemia, elevations are due to one of the following:

(1) Increased production of bilirubin from the breakdown of heme proteins (largely hemoglobin)

(2) Decreased conjugation or excretion of bilirubin

(3) Increased bilirubin enterohepatic circulation (which is less an issue in an older child)

Conjugated hyperbilirubinemia is largely due to either hepatic dysfunction or obstruction of the biliary system. Consideration of jaundice in the older child

requires a different approach than that of a jaundiced neonate (Hsu, 2013). Conjugated hyperbilirubinemia should be suspected in neonates presenting with prolonged hyperbilirubinemia with conjugated bilirubin levels >25 umol/L (Ramachandran, 2015).

Table 2.1: Estimated Occurrence of Neonatal Hyperbilirubinemia Severity (Brits, 2018, Bhutani *et al*., 2004)

TSB (mg/dL)	Proposed Definition	Estimated Occurrence
≥15.0 - <17.0 (250-291 umol/L)	Non-Significant	~1:2
≥17.0 (291 umol/L)	Significant	~1:10
≥20.0 (342 umol/L)	Severe	~1:70
≥25.0 (428 umol/L)	Extreme	~1:700
≥30.0 (513 umol/L)	Hazardous	~1:10,000

TSB, serum total bilirubin

2.2 Catabolism of Heme produces Bilirubin

Two hundred billion erythrocytes are destroyed in human adults per day under normal conditions. Hence, a 70-kg human turns over roughly 6 grams of hemoglobin every day. When hemoglobin is destroyed in the body, globin is degraded to its component amino acids which are reused and the iron of heme goes into the iron pool, also for reuse. Within the reticuloendothelial cells of the spleen,

liver, and bone marrow, the iron-free porphyrin portion of heme is also degraded (Murray, 2012).

The heme catabolism from all heme proteins take place in the cellular microsomal fractions by a complex enzyme system named heme oxygenase. The heme oxygenase system is substrate-inducible. By the period the heme produced from heme proteins arrives the heme oxygenase system, the iron has generally been oxidized to the ferric form, containing hemin. Approximately 1 gram of hemoglobin produces 35 milligrams of bilirubin. About 250-350 mg of bilirubin is formed in adult human, originating chiefly not only from hemoglobin, but also from other various heme proteins for example cytochrome P450 and from ineffective erythropoiesis (Murray, 2012).

Bilirubin are produced from newborns at an amount of 6-8 mg per kg every day. Newborns have beyond double rate of production than in adults, mainly as a consequence of increased erythroid cells change and relative polycythemia in newborns (Porter and Dennis, 2002). Bilirubin is formed by the catabolism of heme in the reticuloendothelial system. Heme is mainly produced from breakdown of red blood cells. Heme in presence of heme oxygenase and biliverdin reductase is converted to bilirubin. Bilirubin is carried to liver bound to plasma protein, albumin. Then, bilirubin is conjugated in liver in the presence of uridinediphospho glucuronosyltransferase enzyme. Conjugated bilirubin is excreted in the bile into the small intestine. Some of the conjugated bilirubin have been converted back to

unconjugated form by the action of beta glucuronidase enzyme present in the small intestine. This process is known as enterohepatic circulation of bilirubin (Ramachandran, 2015). Figure 2.1 showed schematic of bilirubin production and hepatic bilirubin clearance in neonates.

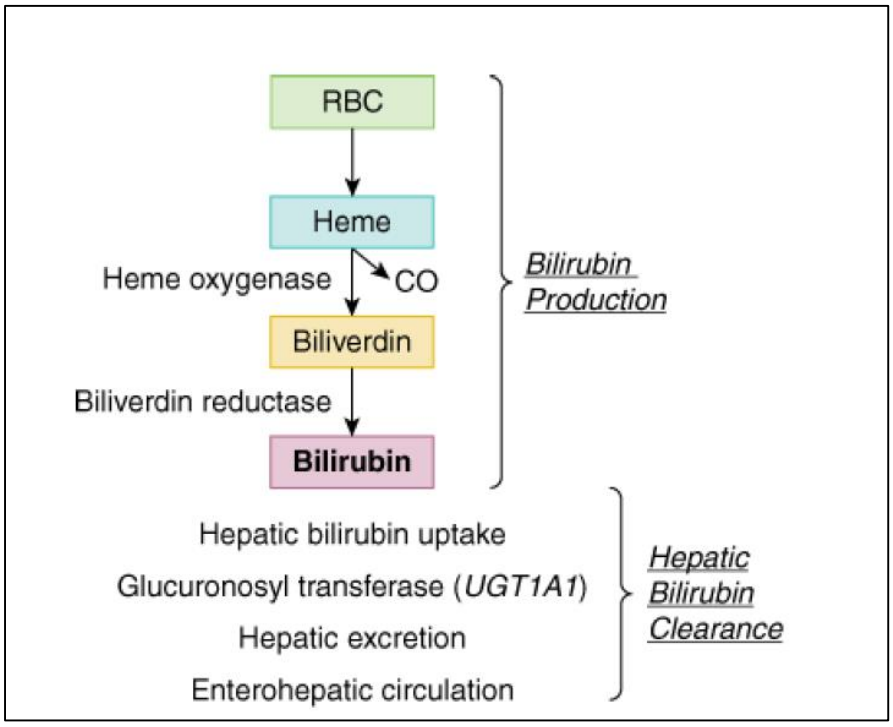


Figure 2.1: Schematic of bilirubin production and hepatic bilirubin clearance in neonates. Heme, produced largely by the breakdown of red blood cells (RBCs), is catabolized by heme oxygenase (HO) to produce an equimolar amount of carbon monoxide (CO) and biliverdin; the latter is reduced to unconjugated bilirubin by biliverdin reductase. Unconjugated bilirubin is taken up by the hepatocyte via facilitated diffusion, bound to glutathione Stransferase (ligandin), and conjugated with glucuronic acid by UGT1A1. Conjugated bilirubin is excreted into bile via multidrug resistance protein 2, a portion of which may be deconjugated by intestinal β-glucuronidases and reabsorbed into the portal circulation enhancing the hepatic bilirubin load (enterohepatic circulation) (Stevenson, 2012)

2.2.1 Differential Diagnosis List of Neonatal Jaundice

Unconjugated Hyperbilirubinemia: Physiologic (Hsu, 2013)

1. **Increased Bilirubin Production**
    - Blood group incompatibility - Rh, ABO, and minor blood group
    - Erythroid cell enzyme abnormalities - pyruvate kinase, glucose-6-phosphate dehydrogenase, and hexokinase deficiency
    - Erythroid cell membrane defect - elliptocytosis, hereditary spherocytosis and pyknocytosis
    - Hemoglobinopathies - α-thalassemia, and sickle cell disease
    - Increased RBC load - cephalohematoma, polycythemia, and ecchymosis
    - Infants of diabetic mothers
2. **Decreased Bilirubin Conjugation or Excretion**
    - Hormonal deficiency - hypothyroidism and panhypopituitarism
    - Bilirubin metabolism disorders- Gilbert syndrome, Crigler-Najjar (type 1 and 2), and Lucey-Driscoll syndrome
    - Sepsis - bacterial, viral, and fungal
3. **Increased Enterohepatic Circulation**
    - Breast-feeding failure jaundice
    - Breast-milk jaundice
    - Bowel obstruction or ileus
    - Pyloric stenosis

2.2.2 Selected Differential Diagnosis Discussion

2.2.2.1 Physiologic Jaundice

Fetal unconjugated bilirubin easily crosses the placenta in utero and is conjugated and excreted by the mother. Following birth, the neonate is solely responsible for bilirubin metabolism. Because of this, jaundice develops in over 50% of newborns in the first 48-72 hours of life. Physiologic jaundice never appears in the first day. Many physiologic factors contribute to this "physiologic hyperbilirubinemia," including a larger RBC mass, decreased RBC life span (70 to 90 days in neonates vs. 120 days in adults), reduced bilirubin uridinediphosphate-glucuronosyltransferase (*UDPGT*) activity (<1% that of adults in the first 10 days of life), and delayed meconium passage leading to increased enterohepatic circulation (Hsu, 2013).

It is the most common type of neonatal jaundice, having no serious complications. Unconjugated bilirubin is the predominant form and its serum level is usually less than 15 mg/dL. Physiologic jaundice usually appears between 24-72 hours of age and peak at 4^{th}-5^{th} days in term neonates and it disappears by 10-14 days of life (Ullah, 2016).

2.2.2.2 Blood Group Incompatibility

If a blood group incompatibility exists between the fetus and the mother, maternal IgG against fetal red cells can cross the placenta, resulting in hemolysis

and increases bilirubin production in the fetus and newborn. An incompatibility can exist in the Rh, ABO, or minor blood group antigens (Hsu, 2013).

2.2.2.3 Rh Incompatibility

Rhesus hemolytic disease of the newborn consequences from maternal red blood cell alloimmunization. Maternal antibodies are produced against fetal red blood cells, when fetus having Rh-positive born to a Rh-negative mother. Maternal immunoglobulin antibodies (IgG) might cross the placenta into the baby circulation and causes mild to severe hemolytic anemia and fetal hydrops (Ullah, 2016).

2.2.2.4 ABO Incompatibility

ABO incompatibility occurs when a mother with type O blood carries a fetus with type A or B blood. This condition is confined to mothers with type O blood because these women carry IgG antibodies (antibodies that react against antigen A and antigen B) that can cross the placenta. Mothers with type A or type B blood produce mostly IgM antibodies against their respective antigens, and these IgM antibodies fail to cross the placenta. Because A and B antigens are common in nature, group O mothers are previously sensitized to these antigens and hemolysis may occur in the first pregnancy (Hsu, 2013).

2.2.2.5 RBC Enzyme Abnormalities

Glucose is the primary metabolic substrate for the erythroid cell. As mature red cell lacks organelles including mitochondria, glucose can only be metabolized

via anaerobic pathways. Defects in glucose metabolism or pathways that protect against red cell oxidation can result in hemolysis and hyperbilirubinemia (Hsu, 2013).

2.2.2.6 Bilirubin Metabolism Disorder

Uridinediphospho glucuronosyltransferase 1A1 (*UGT1A1*) is the key enzyme of bilirubin metabolism. Mutations in the *UGT1A1* gene can cause reduction of *UGT1A1* enzyme activity resulting in an unconjugated hyperbilirubinemia. Type I and II Crigler-Najjar syndrome and Gilbert syndrome subtypes are based on serum bilirubin levels, but believed to be the continuous presentation of a single disease (Abuduxikuer, 2018).

2.2.2.7 Sepsis

Sepsis results in hyperbilirubinemia secondary to hemolysis and impaired conjugation. Patients with sepsis may have both elevated unconjugated and conjugated bilirubin levels. The presence of urinary tract infection has been specifically associated with jaundice presenting after 8 days of age or an elevated conjugated fraction of bilirubin (Hsu, 2013).

2.2.2.8 Breast-Feeding Failure Jaundice and Breast-Milk Jaundice

Many studies have shown a relation between severity of neonatal hyperbilirubinemia and breast-feeding and an increased incidence. Moreover, up to 10 to 30 percentages of breast-fed infants continue to have raised serum bilirubin

levels into the second to sixth weeks of life. Jaundice that happens in the first 2 to 4 days is called “breast-feeding failure jaundice” and that occurring later, from 4 to 7 days and continuing to cause prolonged jaundice, is called “breast-milk jaundice”. There is significant similarity with these two conditions (Hsu, 2013). Hyperbilirubinemia in breast fed neonates usually occurs between 24-72 hours of age (Ullah, 2016).

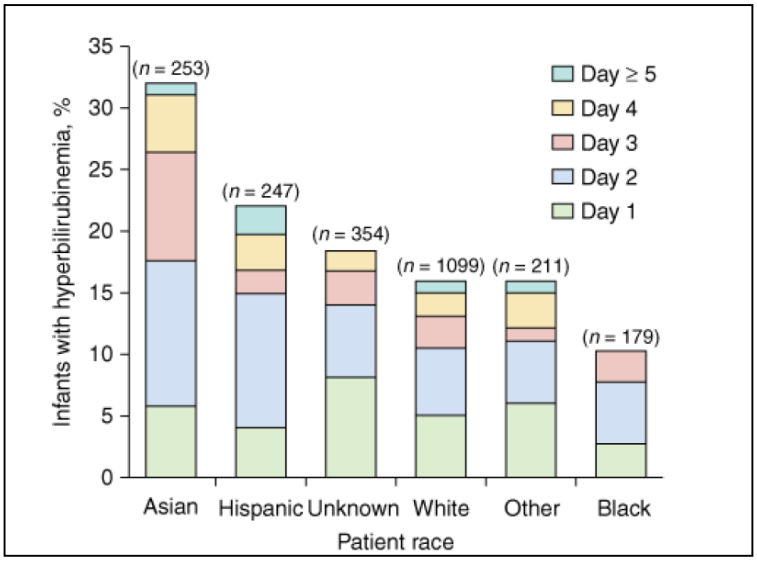


Figure 2.2: Incidence of neonatal hyperbilirubinemia as a function of postnatal age in days and mother’s race. Hyperbilirubinemia was defined as a TSB ≥5 mg/dL (86 µmol/L) at <24 hours of age, ≥10 mg/dL (171 µmol/L) at 24–48 hours of age, or ≥13 mg/dL (222 µmol/L) thereafter. (Newman, 1990)

2.2.2.9 Conjugated Hyperbilirubinemia

The total bilirubin level ≥1 mg/dL or a conjugated fraction that accounts for >20% of the total bilirubin is known as conjugated hyperbilirubinemia. The differential diagnosis of conjugated hyperbilirubinemia, or cholestasis includes both hepatocellular and ductal disturbances of bilirubin excretion (Hsu, 2013). The

common causes are post surgical babies and breast milk jaundice and jaundice related with use of TPN in preterm neonates (Ramachandran, 2015).

2.2.3 Acute Bilirubin Encephalopathy and Kernicterus

Unconjugated bilirubin, in the free or unbound form, that crosses the blood-brain barrier, is toxic to neurons of the basal ganglia and various brainstem nuclei. Clinical manifestations of acute bilirubin encephalopathy are often mistaken for sepsis, asphyxia, or hypoglycemia. In term babies, several phases of acute bilirubin encephalopathy are described (Hsu, 2013). An exact threshold at which kernicterus happens is not possible to define. In well term neonates, risk for kernicterus increases after serum bilirubin levels >340 umol/L (20 mg/dL) and it is very high above 510 umol/L (30 mg/dL) (Ramachandran, 2015).

Data from the Kernicterus Registry suggest an increasing incidence of kernicterus, with 1 case per 624,000 live births in the United States. Some have postulated that early discharge with delayed follow-up, an insufficient response to jaundice by clinicians may play a role in this phenomenon, and failure to check bilirubin levels in neonates with hyperbilirubinemia that presents in the first 24 hours of life (Hsu, 2013).

2.2.4 Treatment of Unconjugated Hyperbilirubinemia

2.2.4.1 Preventive Measures, Nomograms, and Screening Algorithms

Special attention should be paid to infants who are at high risk for developing marked hyperbilirubinemia (see Table 2.2). The preventive strategies include implementation of the existing AAP guidelines and enhancement by a system-based approach (Smitherman, 2006).

Any jaundice occurring in the first 24 hours requires further workup. Additionally, if the clinical assessment suggests significant jaundice, TSB should be checked. Once a TSB is obtained, multiple nomograms exist to assist the clinician with management. The hour-specific bilirubin nomogram developed by Bhutani is helpful in determining a neonate's risk of developing hyperbilirubinemia with the next measurement, and it is used mostly as a tool for determining the appropriate time for follow-up. The AAP has developed treatment nomograms for neonates born at ≥35 weeks of gestation that are stratified by gestational age and the presence or absence of risk factors associated with development of acute bilirubin encephalopathy. The nomograms help the clinician decide whether a neonate should receive phototherapy or exchange transfusion (Maisels *et al*., 2009).

Table 2.2: Important Risk Factors for Severe Hyperbilirubinemia (Hsu, 2013)

Predischarge TSB or TcB measurement in the high-risk or high-intermediate-risk zone
Lower gestational age
Exclusive breast-feeding, particularly if nursing is not going well and weight loss is excessive (>8%-10%)
Jaundice observed in the first 24 hr
Isoimmune or other hemolytic disease (e.g., *G6PD* deficiency)
Previous sibling with jaundice
Cephalohematoma or significant bruising
East Asian race
Hyperbilirubinemia neurotoxicity risk factors
Isoimmune hemolytic disease
G6PD deficiency
Asphyxia
Sepsis
Acidosis
Albumin<3.0 mg/dl

TcB, transcutaneous bilirubin; *G6PD*, glucose-6-phosphate dehydrogenase; *TSB*, total serum bilirubin. Race as defined by mother's description.

Although generally a benign transitional phenomenon with no overt clinical consequences, infants with severe jaundice risked developing serious complications for instance kernicterus (Wong, 2013). Risk factors such as an affected sibling, family history of the condition and East Asian (Chinese, Japanese etc) ancestry suggested that genetic factors are associated (Boo, 2009).

2.2.5 Specific genotypes associated with neonatal hyperbilirubinemia

Genetic variants of uridine diphosphoglucuronate glucuronosyltransferase 1A1 (*UGT1A1*), glucose-6-phosphate dehydrogenase (*G6PD*), hepatic organic anion transporter protein (*OATP2*) heve been reported to be asoociated with neonatal hyperbilirubinemia (Kaplan, 2005). In addition, hemoglobinopathies condition such as alpha-thalassemia is one of the heritable causes of hemolysis which can cause hyperbilirubinemia in neonates (Watchko, 2012, Rets, 2019).

2.2.5.1 *UGT1A1* polymorphisms

Uridine diphosphoglucuronate glucuronosyltransferase 1A1 is the main enzymatic protein for bilirubin conjugation. The *UGT1A1* gene variations have been stated to decrease enzyme activity and be a risk condition for neonatal jaundice. During current decades, the relationship between neonatal jaundice and *UGT1A1* gene polymorphism has been studied. Homozygous variation of the promoter area is associated with neonatal jaundice is found in several studies of Americans, Caucasian populations, including Sephardic Jews, Italians, and British (Kaeda *et al*., 1995).

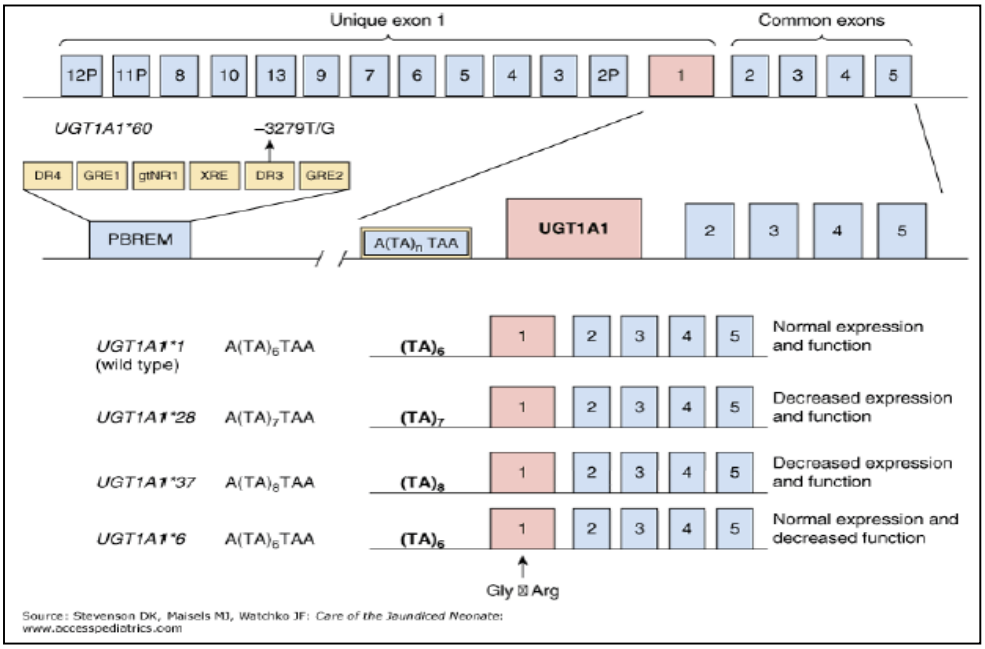


Figure 2.3: Schematic of *UGT1A1* gene. The uppermost panel represents the entire UGT1A gene complex encompassing: (i) the A1 exon, (ii) nine additional exons that encode functional proteins (exons 3–10, 13), (iii) three pseudogenes (exons 2P, 11P, 12P), and (iv) the common domain exon 2–5 sequence shared across all UGT1A transcripts. The UGT1A1 locus and common exons 2–5 are shown in middle panel including the upstream. (Stevenson, 2012)

The *UGT1A1* gene was clarified in 1991 (Ritter *et al*., 1991). Figure 2.3 showed schematic of *UGT1A1* gene. In order to be excreted as the water-soluble form, its product is specific from catalyzing unconjugated bilirubin to conjugate with glucuronic acid. Reduced enzyme activity, which consequences from mutations of this gene, causes the nonhemolytic hyperbilirubinemia of unconjugated fraction known as Gilbert's syndrome and Crigler-Najjar syndrome (Clarke *et al*., 1997).

Uridine diphosphoglucuronate glucuronosyltransferase 1A1 is associated with a broad range of pharmacological, and biological processes because of its important role in the conjugation of a various formation of exogenous, and endogenous compounds. The *UGT1A1* (55 kd, *UGT1A1*_i1) is identical to the 45-kd *UGT1A1*_i2 protein *N*-terminal portion; nevertheless, *UGT1A1*_i2 comprises a unique 10-residue sequence in place of the *UGT1A1*_i1 which has 99-amino acid with C-terminal domain. Western blot and RT-PCR evaluates with a specific antibody against *UGT1A1* show that isoform 2 is differentially expressed in renal, colon, hepatic and small intestine at levels that reach or go beyond, for some tissues, expressed isoform 1. Western blot and immunofluorescence of various cell fractions investigations show that *UGT1A1*_i1 and *UGT1A1*_i2 co-localize in microsomes. Functional enzymatic records designate that *UGT1A1*_i2, this absences transferase action when firmly expressed only in *HEK*293 cells, performances as a negative modulator of *UGT1A1*_i1, reducing its action by 78%. *UGT1A1*_i1 and *UGT1A1*_i2 coimmunoprecipitation proposes that this repression can occur via straight protein-protein connections (Levesque *et al*., 2007).

A key phase II conjugation pathway represented as glucuronidation, catalyzed by *UGT1A1* enzymes, and is implicated in the metabolism and excretion of numerous endogenous compounds. The endoplasmic reticulum is the site for uridine glucuronosyltransferase protein, and this enzyme transfers the glucuronic acid. This process is involved in the elimination of steroids, bile acids, bilirubin,

toxic dietary components, and numerous drugs, such as irinotecan, morphine, and mycophenolate mofetil (Bullingham *et al*., 1998).

Four families are included in the family of *UGT* gene (Mackenzie *et al*., 2005). The structures of *UGT1* and *UGT2* have been characterized in humans over the last few years; together, 16 functional proteins are encoded by these genes. *UGT1A* encodes 9 functional *UGT1A* proteins, and includes 17 exons, is situated on chromosome 2q27. The amino-terminal half of the protein that imparts aglycone specificity is encoded by each unique exon 1 (Gong *et al*., 2001).

In 1991, the cDNA of human UDP-glucuronosyltransferase 1A1 gene was cloned (Kuwahata *et al*., 2010). At least 113 mutations or variants of the *UGT1A1* gene have been identified (Canada Research Chair in Pharcogenomics). The genetic defects in subjects with mild hyperbilirubinemia Gilbert's syndrome, severe hyperbilirubinemia type 1 Crigler-Najjar syndrome, moderate hyperbilirubinemia type 2 Crigler-Najjar syndrome were identified in 1992, 1993 and 1995 respectively (Bosma *et al*., 1995, Aono *et al*., 1995).

The coupling of hypomorphic *UGT1A1* coding and promoter sequence variants would be expected to reduce *UGT1A1* isoenzyme activity further and thereby enhance neonatal hyperbilirubinemia risk. Coexpression of *UGT1A1* promoter and coding sequence variants merits further study as contributor to the higher occurrence of jaundice in East Asian infants (Watchko and Lin, 2010).

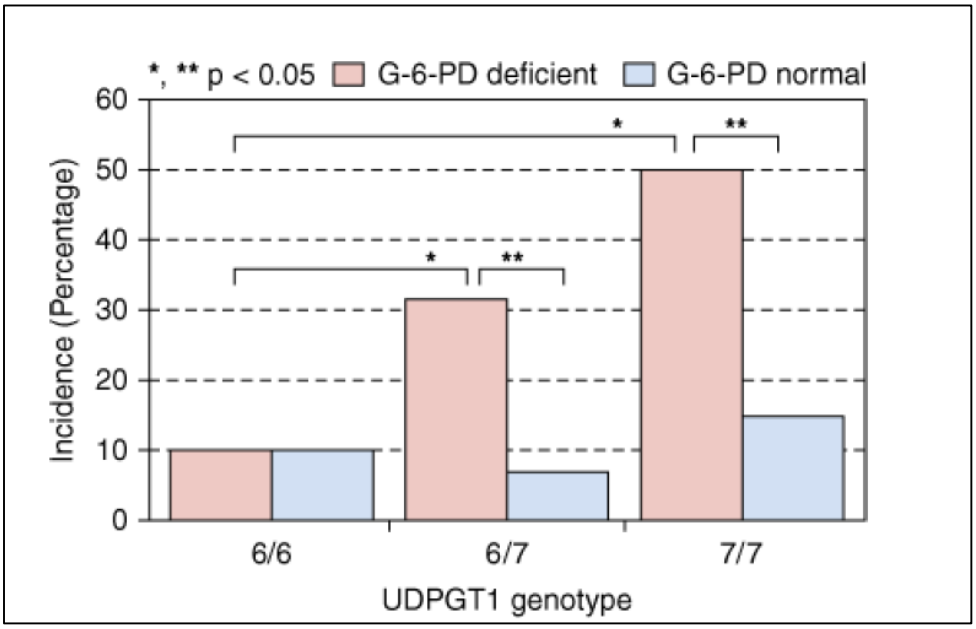


Figure 2.4: Hyperbilirubinemia (TSB >15 mg/dL [257 µmol/L]) incidence in G6PD-deficient (G6PD Mediterranean mutation) neonates and normal controls as a function of UGT1A1*28 promoter genotype. (Stevenson, 2012)

The study by Zhou *et al*., 2014 reported that breast-fed hyperbilirubinemia was associated with five significant associations, such as gender, delivery mode, body weight of a baby at its birth, gestational age and False Discovery Rate correction: the dominant effect of variant $(TA)_n$ promoter and rs4148323 (G211A), the additive effect of (TA) repeat, the recessive effect of rs6719561 (2558T>C). In East Asian population, their findings added to the neonatal hyperbilirubinemia in association with the significance of *UGT1A1* mutations. This study suggested that other evaluations were essential to explore the pathogenesis of the protective effect.

Hanchard *et al*., 2011 stated that three new coding mutations were detected, but there was no strong association between bilirubin rise and rare defects. Adjusted

linear regression models suit to assess the association among common *UGT1A1* variants and changing bilirubin levels in 39 neonates, found that patients carrying the wild-type had significantly larger changes in bilirubin than subjects with homozygous mutation ($p = 0.003$).

A new finding reported by Saab *et al*., 2013, that is, *UGT1A1* TA promoter variety not only reflects geographic distribution but also interethnic differences as well. Variants *UGT1A1**28 with decreased-function were common in Southern Asian and African populations in particular. The greatest incidence of the active gene was detected in South East and East Asian peoples. This finding showed that developing pharmacogenetic biomarkers must be considered between diverse ethnic people.

2.2.5.2 Glucose-6-phosphate dehydrogenase mutations

Glucose-6-phosphate dehydrogenase deficiency is one of the most frequent genetic diseases known in newborns with extreme hyperbilirubinemia and it is related with severe hemolysis and bilirubin encephalopathy (Beutler, 1994, Cappellini and Fiorelli, 2008, Luzzatto, 2006, WHO working group, 1989). The disorder has X-linked recessive inheritance and occurs with high frequency among persons of Africans, Mediterranean, and Asian ancestry. Hundreds of different *G6PD* variants have been characterized. The severity of the disorder varies among ethnic groups; *G6PD* deficiency in persons of African ancestry usually is less severe than in other ethnic groups (William *et al*., 2012).

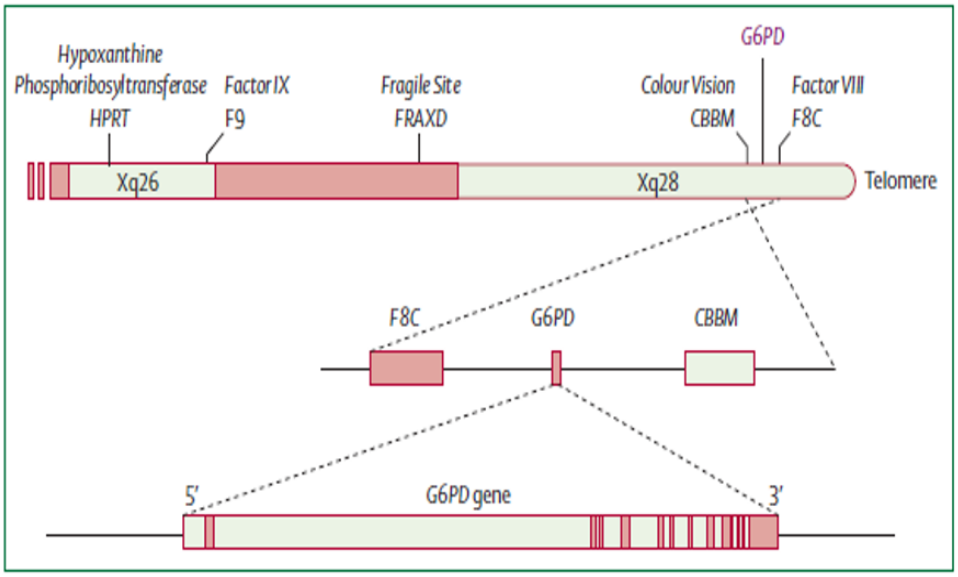


Figure 2.5: Location of *G6PD* gene on X chromosome. *G6PD* gene is located at long arm of the X chromosome (band Xq28), close to the genes for haemophilia A, congenital dyskeratosis, and colour blindness (Cappellini, 2008)

The *G6PD* enzyme involves the foremost step in the phosphogluconate pathway and its function has been well described and it is accountable for the formation of NADP to NADPH, which acts as hydrogen ion donor. These hydrogen ions contribute to the alteration of oxidized form of glutathione to its reduced form and the stability of an important antioxidant catalase (Kaplan and Hammerman, 2010). Worldwide, 4.9% of global population or 330-million individuals estimated to affect *G6PD* deficiency (Nkhoma *et al*., 2009).

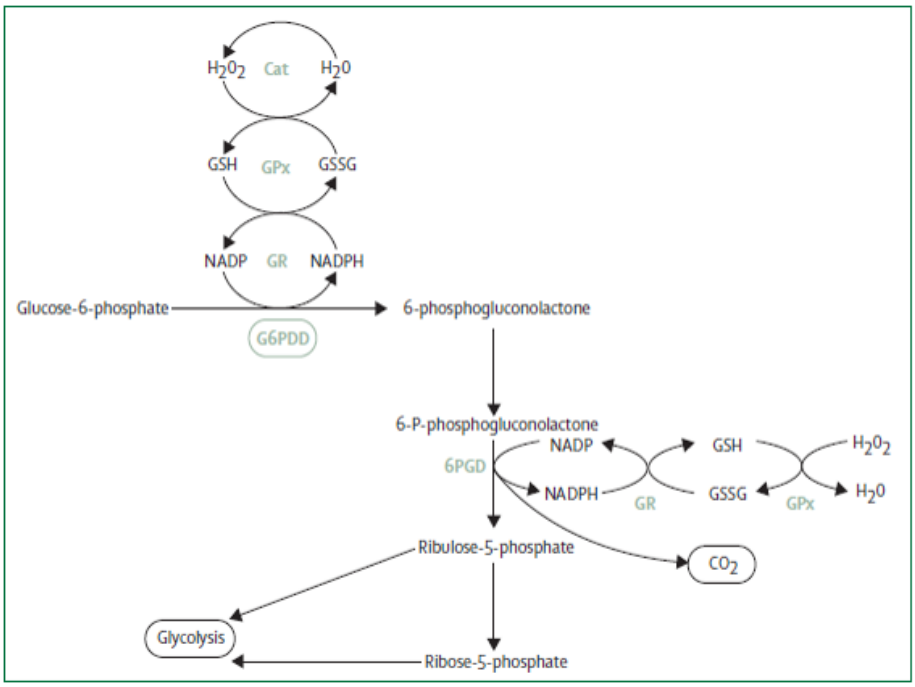


Figure 2.6: The Pentose Phosphate Pathway. NADPH is produced by the action of G6PD and 6-phosphogluconate dehydrogenase. It serves as a proton donor for the regeneration of reduced glutathione, and as a ligand for catalase. NADPH also acts as an electron donor for many other enzymatic reactions essential in reductive biosynthesis. Cat=catalase. GPX=glutathione peroxidase. GR=glutathione reductase. G6PDD=glucose–6–phosphate dehydrogenase. 6PGD=6-phosphogluconate dehydrogenase. GSH=reduced glutathione. GSSG=oxidised glutothione. (Cappellini, 2008)

Infants with *G6PD* deficiency may have significant hyperbilirubinemia and require exchange transfusion or phototherapy to prevent kernicterus. The disorder is a main etiology of neonatal jaundice in infants of Asian or Mediterranean ancestry, but less in infants of African ancestry. Ingestion of fava beans may trigger hemolysis in children of Mediterranean or Asian ancestry but usually not in children of African ancestry. Episodes of hemolysis are associated with pallor, jaundice, hemoglobinuria, and sometimes cardiovascular compromise (William *et al*., 2012).

Over 300 *G6PD* variants have been identified. Acute intermittent hemolytic anemia is associated with most common variants; some of these variants are endemic (some Southeast Asian variants, Mediterranean, A^-). In contrast, chronic hemolytic anemia associated variants are very rare. The other type of variant is associated with no apparent hazard of hemolysis and is also uncommon (Hoffman, 2012).

Based on clinical manifestations and enzyme activity, glucose-6-phosphate dehydrogenase deficiency variants were grouped into five classes (Table 2.3) (WHO working group, 1989).

Table 2.3: *G6PD* deficiency classes (WHO working group, 1989)

Class I Severely deficient, associated with chronic non-spherocytic hemolytic anaemia
Class II Severely deficient (1-10% of residual activity), associated with acute hemolytic anaemia
Class III Moderately deficient (10-60% residual activity)
Class IV Normal activity (60-150%)
Class V Increased activity (>150%)

2.2.5.2.1 Polymorphic variants – malaria selection

It is recognized that the occurrence of polymorphic mutations has increased because *G6PD* deficiency causes relative defense against severe malaria (Allison, 1960, Motulsky, 1960). Since massive amounts of oxidizing agents are produced by red cells infected by malaria because hemoglobin is reduced in quality in the parasite food vacuole, *G6PD* deficient red cells are engulfed and ingested, and quickly destroyed together with their malarial parasites (Saunders *et al*., 2005). The recent study (Rets *et al*., 2019) stated that the two most common polymorphic variants are *G6PDA*- and *G6PD*-Mediterranean.

In Malaysia, the commonest types reported were *G6PD* Mahidol (G487A) and *G6PD* Vianghan (G871A, IVS11 nt 93 T>C, C1311T) and Indo-Pakistan subtype (C563T, IVS11 nt 93 T>C, C1311T) and *G6PD* Mediterranean: Mediterranean subtypes (IVS11 nt 93 T>C, 563 C>T) are also detected. *G6PD* Canton (G1376T), *G6PD* Kaiping (G1388A), (Ainoon *et al*., 1995) and *G6PD* Gaohe (95 A>G) are common in Chinese background and Malaysian Indian had Indo-Pakistan subtype of *G6PD* Mediterranean. *G6PD* Namoru (T208C) were also detected in Malaysia (Wang *et al*., 2008). The study by Aninoon reported that the molecular defects of *G6PD* deficiency was diverse in Malays and 82.6% of *G6PD*-deficient neonates had neonatal jaundice. *G6PD* Viangchan, Mediterranean, and Mahidol comprise as a minimum 80% of patients (Ainoon *et al*., 2003).

Likewise, Wang *et al*., 2009 studied by using *G6PD* enzyme assay, fluorescent spot test, and SNPs study of the *G6PD* gene, where five of seven frequent variants identified to occur in Malaysian Chinese (G871A, G1376T, A95G, G1388A, C1024T, G563T, G487A) were detected in which the three variants (1388 G>A, 1376 G>T and 871 G>A) were associated with severe hyperbilirubinemia. In the same report, 19.6% of neonates (39 of 199) were observed to have *G6PD* deficiency by enzymatic assay. The study by Yusoff *et al*., 2003 reported that *G6PD* Mediterranean and Viangchan constitute the main mutations in Malays.

The frequency of *G6PD* deficiency has spread broadly from its population origins in tropical malaria-laden latitudes to a global distribution (Watchko and Lin, 2010).

2.2.5.2.2 Sporadic variants – chronic nonspherocytic hemolytic anaemia

Sporadic mutations initiating chronic nonspherocytic hemolytic anaemia (CNSHA, also called WHO Class I) happen in any part of the world at a very low frequency. Single amino acid changes are associated with majority of mutations (Mason *et al*., 1995, Vulliamy *et al*., 1998). Sporadic mutations have seen in areas wherever polymorphic mutations are detected too in a few cases but producing the two phenotypes with dissimilar amino acid changes (Mason *et al*., 2007).

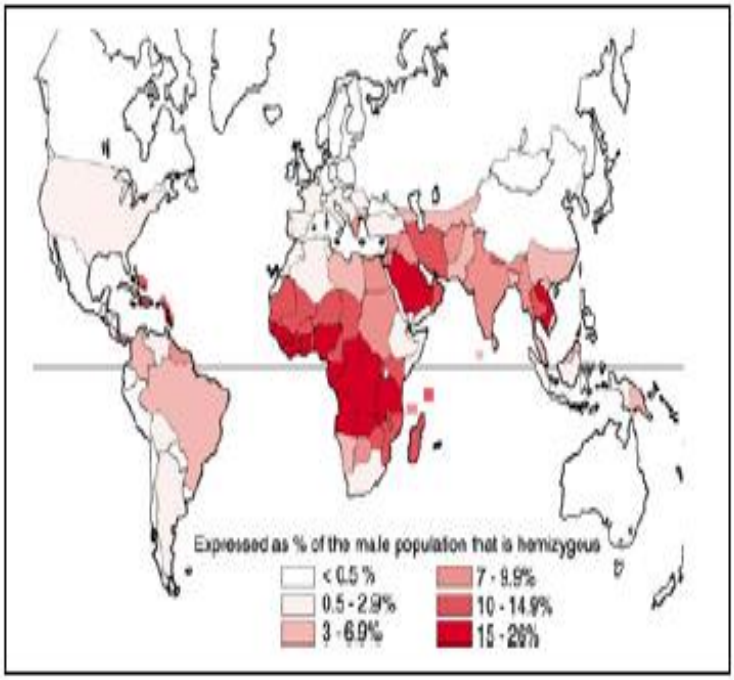


Figure 2.7: Distribution of glucose-6-phosphate dehydrogenase deficiency. G6PD deficiency is quite prevalent in the Africa, Asia, Middle East and Mediterranean and present in several forms throughout the world. (WHO Working Group, 1989)

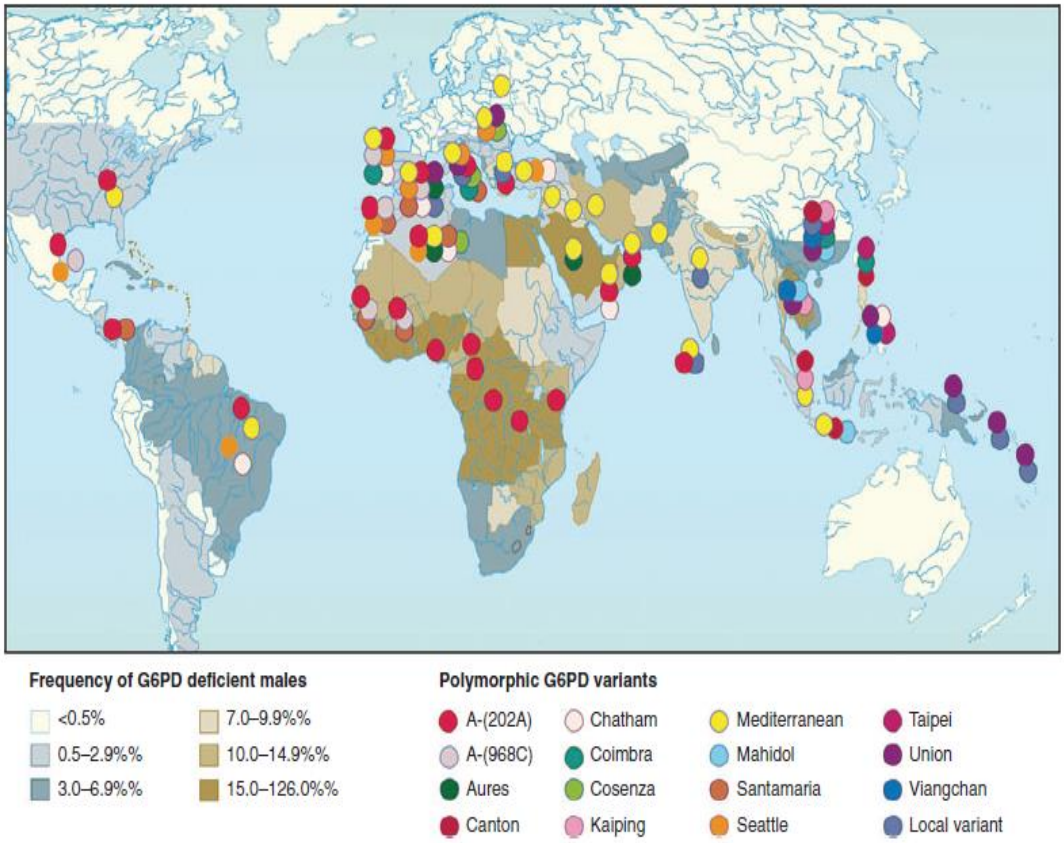


Figure 2.8: Glucose-6-phosphate dehydrogenase mutations distribution. World map of G6PD defi ciency: the shadings indicate the overall prevalence of G6PD defi ciency in individual countries; the distribution of individual G6PD variants is shown by colored symbols. (Luzzatto, 2006)

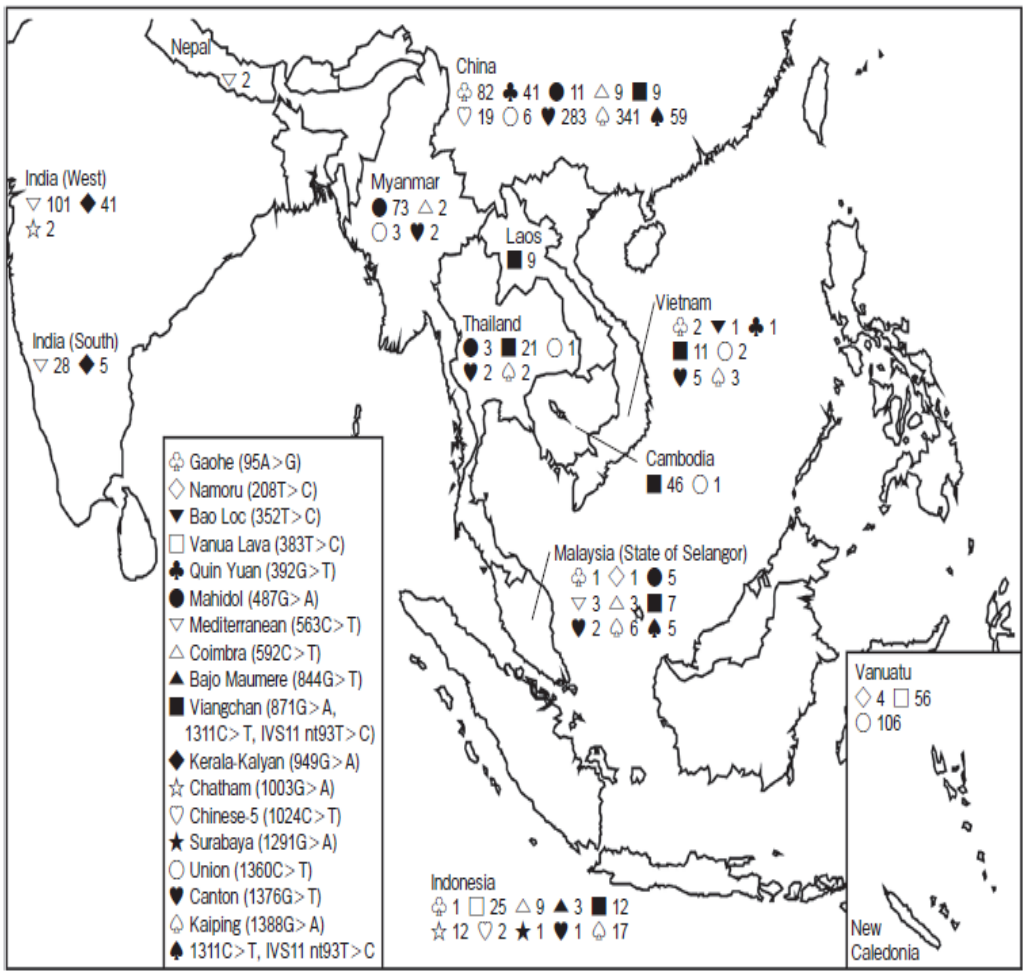


Figure 2.9: Distribution and frequencies of glucose-6-phosphate dehydrogenase (G6PD) variants in Asian countries and Vanuatu, Melanesia. Each number indicates the number of G6PD-deficient cases confirmed by sequence analysis. (Wang et al., 2008)

2.2.5.2.3 Biochemical Genetics

Glucose-6-phosphate dehydrogenase is designated as the normal or wild-type enzyme. Many variants of *G6PD* were identified, related with phenotypes and an extensive range of biochemical appearances. *G6PD* variants were differentiated from each other based on biochemical natures, such as capacity to use substrate

analogues, K_m for NADP and pH activity profile, electrophoretic mobility, and thermal stability (Williams, 2016).

2.2.5.2.4 Epidemiology

Several variants are pandemic in Asia. *G6PD* Mahidol type is frequently observed in Thai and India while *G6PD* Chinese-1, *G6PD* Chinese-2, *G6PD* Chinese-3, and *G6PD* Canton are frequent in China and Southeast Asia. *G6PD* Viangchan (G871A) is the most frequent allele in Cambodia and also found in Thais and Laotians. It is assumed that malaria provided a positive selection pressure, accounting for the high gene frequency of these *G6PD* variants, whereas mutations that arose in parts of the world not plagued by malaria are sporadic (Hoffman, 2012).

2.2.5.2.5 African Variants

A mutant *G6PD A+* enzyme, with normal activity is polymorphic among people of African descent. Among people of African origin, *G6PD A-* is the key deficient variant detected (Williams, 2016).

2.2.5.2.6 Variants in the Mediterranean Region

Among white people, *G6PD* deficiency is most commonly found in Mediterranean regions. *G6PD* Mediterranean type is the most frequent in this area. This abnormal gene is hardly detectable in individuals who have inherited with the enzyme activity of the red cell (Williams, 2016).

2.2.5.2.7 Variants in Asia

In Asian populations, huge varied diverse mutations were identified. Some of these variants are very identical (e.g., *G6PD* Canton, Agrigento, Taiwan-Hakka, and Gifu). Molecular studies have revealed that many different mutations are detected in various Asian people (Williams, 2016).

2.2.5.2.8 Variants Producing Hereditary Nonspherocytic Hemolytic Anemia

Few variants of *G6PD* defects consequence in chronic hemolysis without having precipitating causes. These variants are listed as class I mutants. These conditions are more severe than the polymorphic types for example *G6PD A-* and *G6PD* Mediterranean, from a functional point of view, but the enzymatic action can be higher in these variants *in vitro* (Williams, 2016).

2.2.5.2.9 Acute Hemolysis and *G6PD* Deficiency

Hemoglobin is maintained in solution in a high concentration in the cytoplasm of the erythrocyte. The oxidation of free –SH groups of hemoglobin is due to oxidant damage. The precipitated hemoglobin may be morphologically acknowledged as Heinz bodies. The increase ratio of reduced-to-oxidized glutathione represents the main defense against the hemoglobin oxidative damage. The *G6PD* enzymatic activity produces NADPH that is utilized for glutathione

reduction. Reduced glutathione (GSH) reconstitutes –SH groups of hemoglobin, maintaining the solubility of hemoglobin (Hoffman, 2012).

2.2.5.2.10 Drug-Induced Hemolysis

Drug-induced hemolysis is usually associated with the development of stromal protein, and tiny pieces of denatured hemoglobin, Heinz bodies. These Heinz bodies inclusions has been the topic of appreciable speculation and investigation (Williams, 2016).

2.2.5.2.11 Infection-Induced Red Cells Hemolysis

The pathogenesis of erythroid cells lysis in infection is unknown. The production of hydrogen peroxide by phagocytizing white blood cells can possibly be part of pathogenesis (Williams, 2016).

2.2.5.2.12 Favism

Fava beans are a main risk food in several areas where *G6PD* deficiency is identified at a high gene frequency. The hemolysis precipitated by favism, fava bean ingestion, has been widely recognized in the Mediterranean region and occurs only in subjects who are also *G6PD* deficient. However, not all *G6PD*-deficient variants are prone to favism (Hoffman, 2012).

2.2.5.2.13 Molecular Biology

The *G6PD* gene is situated on the long arm of X-chromosome. It extends over 18kb and consisting 13 exons. The coding sequence starts in exon 2. The promoter shares several common features of other housekeeping genes. The enzymatic protein is possessed of 515 amino acids with a molecular weight of about 59 kDa. NADP is bound to the enzymatic protein together as one of the substrates of the reaction and as a structural component (Williams, 2016).

2.2.5.2.14 Neonatal Jaundice and *G6PD* deficiency

Some infants with *G6PD* deficiency are associated with neonatal jaundice without having immunopathogenesis. Indeed, it has been proposed that nearly 30% of all male infants with hyperbilirubinemia may be associated with *G6PD* deficient condition. Clinical condition can be more severe in premature newborns. The jaundice can result in kernicterus if untreated. Therefore, *G6PD* deficiency can cause brain damage, and this aspect of the condition has significant public health consequence. Hence, *G6PD* deficiency should be tested in newborns with sever neonatal hyperbilirubinemia. The condition is frequently related with severe red cells hemolysis (Williams, 2016).

2.2.5.2.15 Clinical Features

Subjects with the frequent polymorphic forms of *G6PD* mutations, such as *G6PD* Mediterranean or *G6PD A-*, generally have no obvious clinical appearances. The main sequelae of *G6PD* enzyme defect are neonatal jaundice in infants and

hemolytic anemia in adults. The anemia is usually intermittent, but certain forms of the uncommon *G6PD* variants may result in congenital nonspherocytic hemolytic condition (Williams, 2016).

2.2.5.2.16 Evolutionary Benefit of *G6PD* Deficiency

Shortly after the discovery of *G6PD* deficiency, it became apparent that the prevalence of malaria overlapped closely with the geographical distribution of people. On the basic of association, it was suggested that *G6PD* deficiency might be shielding of malarial infection. Unexpectedly, in the initial studies of acutely infected subjects, no protective effect of the *G6PD* deficiency was found for hemizygous *G6PD*-deficient males. However, an apparent protection was found for heterogygous females who, because of X-chromosome inactivation, are mosaics for deficient and nondeficient cells. This led to elegant studies demonstrating that although the malaria parasite does not initially thrive in a *G6PD*-deficient environment, it adapts after several days of residence in a *G6PD*-deficient erythrocyte (Hoffman, 2012).

2.2.5.2.17 Diagnosis

The *G6PD* deficiency diagnosis is based on the generation of NADPH as detected by either quantifiable colorimetric method or, more conveniently, by a rapid fluorescent screening method. False-negative results are not uncommon, particularly if enzymatic analysis is performed soon after resolution of acute hemolytic episodes. Young red cells and reticulocytes, which have much greater

enzymatic activity, predominates after acute hemolysis. The false-negative test results are more possible to occur when a screening test rather than a quantitative spectrophotometric analysis of the enzyme activity is used. A high proportion of young red blood cells, which could contribute to a falsely "normal" result, can be conveniently estimated by concomitant spectrophotometric analysis of hexokinase, another red blood cell enzyme whose activity is markedly dependent on age of erythrocyte (Hoffman, 2012).

Laboratory Findings

Various degree of reticulosis and anemia are the key findings of individuals with congenital hemolytic anemia. Heinz bodies formations are frequently detected in the red cells especially in drug-induced red cells lysis. Sometimes, the light-microscopic feature of red cells appears to be normal. Fragments of red cell formation and spherocytosis can be observed in the peripheral blood film if the hemolytic anemia is very severe. Even though the "bite cells" are found in the blood film of a patient associated with hemolysis after drug exposure, the pathogenesis is uncertain since these cells are generally not found in acute hemolysis of *G6PD*-deficient individuals with chronic hemolysis or subjects with in common *G6PD* variants mutation. Moreover, "bite cells" have also been found in *G6PD* nondeficient individuals (Williams, 2016).

More than 160 various mutations of the *G6PD* gene have been found (Beutler and Vulliamy, 2012), and over 400 biochemical variants have been

identified based on various conditions such as kinetic variables, pH dependence, and physicochemical properties (Beutler, 1984). Variants can be classified as polymorphic or sporadic (Chen *et al*., 2008). Different polymorphic variants have been set for different geographical regions (Ainnon *et al*., 1995).

Furthermore, combination of *G6PD* deficiency and *UGT1A1* genetic polymorphisms may result in greater incidence of neonatal jaundice, usually responsive to phototherapy treatment but occasionally needing exchange transfusion. Moreover, combination of *G6PD* deficiency, *UGT1A1* genetic polymorphisms and environmental influences may upset bilirubin production-conjugation equilibrium, precipitate severe, sudden and exponential increase in serum bilirubin with potential for bilirubin encephalopathy (Kaplan and Hammerman, 2010).

Hence, *G6PD* deficiency is very frequent not merely in developing nations but also encountered in numerous industrialized nations (Kaplan and Hammerman, 2010). Patients with *G6PD* deficient condition cause acute hemolytic anaemia or neonatal jaundice, which, if treated ineffectively, can develop permanent brain damage or death (Cappellin and Fiorelli, 2008). Glucose-6-phosphate dehydrogenase deficiency is not only related with major etiologic factor in the pathogenesis of bilirubin encephalopathy but also significant neonatal hyperbilirubinemia (Riskin *et al*., 2012).

Kaeda *et al*., 1995 study reported that, in tribal Indian populations, *G6PD* Orissa (Ala44Gly) is accountable for most of the cases but is not detected in urban group, where G6PD Mediterranean (188 Ser>Phe) is the most common form of variant.

In Thailand, the study group by Nuchprayoon reported that the *G6PD* deficiency prevalence in males is 22.1% and 10.1% in females, 871 G>A was the most common variant identified, after that 1376 G>T, 487 G>A, 1388 G>A, 1024 C>T and 1360 C>T. This study concluded that 871 G>A (*G6PD* Vianchan) is possibly the most frequent mutation in Southeast Asian people of non-Chinese origin (Nuchprayoon *et al*., 2002).

Another study of Nuchpranoon *et al*., 2008 done in Thailand Samutsakhon province stated that, in migrated male populations of Mon and Burmese of southern Myanmar, *G6PD* deficiency was found 12% (19 of 162) in Mon males and 10% (17 of 178) Burmese male subjects. Among *G6PD*-deficient Mon males (n=19), 12 cases were G487A (*G6PD* Mahidol); one case each was C563T (*G6PD* Mediterranean), G1388A (*G6PD* Kaiping), G871A (*G6PD* Jammu), a novel mutation C94G; and three remain unidentified. Among *G6PD*-deficient Burmese males (n=17), 12 cases were G487A (*G6PD* Mahidol); one each was C406T (*G6PD* Valladolid), G949A (*G6PD* Kerala-Kalyan), C592T (*G6PD* Coimbra), and two cases remain unidentified. G487A (*G6PD* Mahidol) is the most common variant among Burmese and Mons.

In Southeast Asia, the study by Hamada identified common 11 mutations of *G6PD* and the distribution of mutations were G1376T (*G6PD* Canton; 24.0%), G871A (*G6PD* Viangchan; 18.0%), G1388A (*G6PD* Kaiping; 11.0%), C563T (*G6PD* Mediterranean; 6.0%), G592A (*G6PD* Gaohe; 6.0%), G487A (*G6PD* Mahidol; 5.0%), C131G (*G6PD* Orissa; 4.0%), C592T (*G6PD* Coimbra; 3.0%) and G1003A (*G6PD* Chatham; 2.0%), respectively. The unidentified 8 samples were detected by direct sequencing to carry four unknown mutations such as G392T (*G6PD* Quing Yuan; 4.0%), C1024T (Chinese-5; 2.0%), T517C (*G6PD* Nankang; 1.0%) and A209G (*G6PD* Murcia; 1.0%) (Hamada *et al*., 2010).

Phompradit *et al* study done in Burmese and Thai peoples stated that almost all of *G6PD* mutations sample taken from Burmese subjects have G487A (96.2%) variant and only one sample has G1388A (3.8%) allele. In Thai subjects, the most common variant detected was G487A (*G6PD* Mahidol; 38.1%), followed by G871A (*G6PD* Viangchan; 19.0%), *G6PD* Chinese 4 (14.3%), G1376T (*G6PD* Canton; 9.5%), G592A (*G6PD* Gaohe; 4.8%), and G1388A (*G6PD* Kaiping; 4.8%) (Phompradit *et al*., 2011).

The study by Li., 2015 reported the prevalence of *G6PD* deficiency in the Jingpo Kachin racial group in the Myanmar-China border region was 29.6% (523 of 1770), among which 30.6% and 27.9% were females and males respectively. From these samples, the most common mutation was G487A (*G6PD* Mahidol;

89.7%), followed by G1388A (*G6PD* Kaiping; 8.0%), and G871A (*G6PD* Viangchan; 2.2%) variants. The *G6PD* Chinese 4 and *G6PD* Canton variants are found in 1.1% each.

Moiz *et al*., 2012 study reported that *G6PD* variant C563T were detected in *G6PD* deficient newborns in Pakistan. This study concluded that patients with *G6PD* C563T variant caused hyperbilirubinemia prior to subjects with normal enzymatic protein levels. *G6PD* nt C563T carrying newborns had considerably reduced enzyme function compared to normal level newborns.

In Malaysia, Ainoon *et al* stated that the prevalence of *G6PD* deficiency were G871A (*G6PD* Viangchan; 37.2%), C563T (*G6PD* Mediterranean; 26.7%) and G487A (*G6PD* Mahidol; 15.1%) followed by G1376T (*G6PD* Canton; 4.7%), T383C (*G6PD* Vanua Lava; 3.5%), C592T (*G6PD* Coimbra; 3.5%), G1388A (*G6PD* Kaiping; 2.3%), C1360T (*G6PD* Union; 2.3%), G1003A (*G6PD* Chatham; 2.3%), C131G (*G6PD* Orissa; 1.2%) and G1361A (*G6PD* Andalus; 1.2%), respectively (Ainoon *et al*., 2002).

Another study by Ainoon *et al*., 2004 done in Malaysian Chinese male neonates reported that the two commonest alleles were G1376T (*G6PD* Canton; 42.3%) and G1388A (*G6PD* Kaiping; 39.4%) followed by G592A (*G6PD* Gaohe; 7.0%), T517C (*G6PD* Nankang; 1.5%), C1024T (*G6PD* Chinese-5; 1.5%), G487A (*G6PD* Mahidol; 1.6%), G871A (*G6PD* Viangchan; 0.8%), C1360T (*G6PD* Union;

0.8%), G392T (*G6PD* Quing Yang; 0.8%), and G1003T (*G6PD* Chatham; 0.8%), 68.0% (88 of 125) neonates in this study had neonatal jaundice and 29.7% developed jaundice with TSB >250 umol/l. The incidence of jaundice >250 umol/l was higher in G1388A (*G6PD* Kaiping; 43.8%) than G1376T (*G6PD* Canton; 22.0%) ($p< 0.05$). There was no significant difference in the mean age for peak serum total bilirubin, mean serum total bilirubin, incidence of neonatal hyperbilirubinemia, mean duration of phototherapy between the two major variants and percentage of newborns requiring phototherapy.

Table 2.4: Results of Malaysia *G6PD* Deficiency in newborns showing the prevalences of G6PD variant mutations

G6PD Variants	G6PD Studies (Malaysia)						
	Ainoon *et al.*, 1995	**Ainoon *et al.*, 1999**	**Ainoon *et al.*, 2002**	**Yusoff *et al.*, 2003**	**Ainoon *et al.*, 2004**	**Wang et al., 2008**	**Amini *et al.*, 2011**
Viangchan			37.2%	35.4%	0.8%	22.2%	12.0%
Kaiping		34.2%	2.3%	7.7%	39.4%	37.3%	
Mahidol			15.1%	9.2%	1.6%	11.1%	
Canton	61%	50.0%	4.7%	1.5%	42.3%	7.4%	
Mediterranean			26.7%	16.9%		11.1%	
Orissa			1.2%	3.1%			
Chatham			2.3%	1.5%	0.8%		
Vanua Lava			3.5%	18.5%			
1311C>T						18.5%	64.0%
Union			2.3%		0.8%		
Chinese-5		2.2%			1.5%		
Gaohe		5.2%			7.0%	3.7%	
Coimbra			3.5%	6.2%		3.7%	4.0%
Namoru						3.7%	
Nankang					1.5%		
Qing Yang					0.8%		
Unidentified		7.0%		14.0%			20.0%

Table 2.4 shows previous some studies done on Malaysian *G6PD* deficient neonates with their findings. The most common *G6PD* variants detected in Malaysian newborns from these studies were selected for the study. In addition, no

studies have been reported on the most common *G6PD* variants associated with significant hyperbilirubinemia in Malaysia.

2.2.5.3 *SLCO1B1* polymorphisms

Numerous substances are excreted from the liver, and the liver sinusoidal membrane is composed of transport proteins that facilitate efflux and influx of these substances. In humans, *OATP* transport proteins take part in the foremost phase of hepatic excretion by assisting hepatic uptake (Abe *et al*., 1999). Organic anion transporter polypeptide 2 (*OATP2*) enzymes is responsible for cellular uptake of bilirubin and play a significant role in regulating bilirubin concentrations (Liu *et al*., 2013, Rets, 2019).

Hence, *OATP* transporters genetic polymorphisms have important indications for management. As a minimum eight *OATP* family members are identified in humans, i.e., *SLC21A3, SLC21A9, SLC21A6, SLC21A11, SLC21A12, SLC21A14,* the prostaglandin transporter *PTG (SLC21A2),* and *SLC21A8 (OATP-8).* From these, *SLC21A9, SLC21A6, SLC21A11, SLC21A12,* and *SLC21A8* are expressed in liver and involved in significant parts in hepatic handling of xenobiotics, for instance benzylpenicillin, and endogenous substances such as leukotrines, steroid hormone conjugates, and bilirubin (Tamai *et al*., 2001, Cui *et al*., 2001). Figure 2.10 showed schematic of *SLCO1B1* gene.

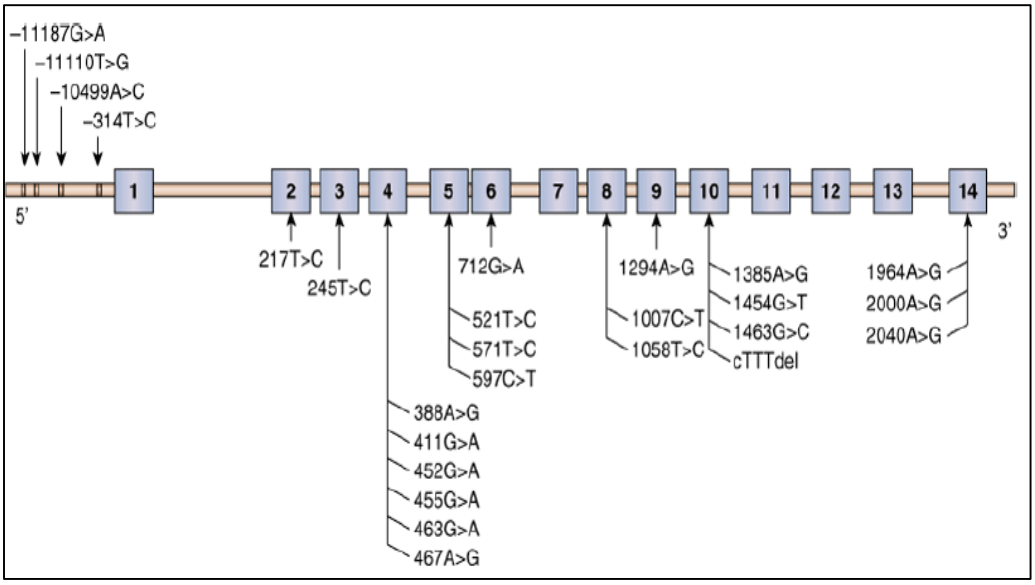


Figure 2.10: Schematic of *SLCO1B1* gene and identified polymorphisms in promoter (above) and coding (below) sequences. The 388A>G nonsynonymous polymorphism (SLCO1B1*1b) has been reported in association with significant neonatal hyperbilirubinemia in some populations. (Stevenson, 2012)

The *SLC21A6* sequence has been labeled as *OATP-C**1a. The SNPs 521 T>C (V174A) and 388 A>G (N130D) are located on the *OATP-C* gene. The *OATP-C**1b cDNA sequence was also identified. The *SLC21A9 (OATP-B)* sequence was designated as *OATP-B**1 with the accession number AB026256 (Nozawa *et al*., 2002).

The coexpression of *SLCO1B1* and/or *UGT1A1* variants involves a clinically significant part in modulating risk of neonatal jaundice in African American newborns. This is supported by the observation that the occurrence of African *G6PD* A- mutation only is not associated with an increased risk of marked

hyperbilirubinemia in a large cohort of African American newborns (Watchko and Lin, 2010).

Coexpression of *G6PD* with *SLCO1B1* variants mutations has not been described for neonates but would be predicted to increase their hyperbilirubinemia hazard. Indeed, homozygous *SLCO1B1* nucleotide A388G variant mutation significantly increased the risk for a total serum bilirubin level of >20 mg/dL (OR: 3.02 [95%CI: 1.30-6.99]) (Watchko *et al*., 2009).

Coexpression of *UGT1A1* with *SLCO1B1* variants in newborns with hyperbilirubinemia who also carry *G6PD* mutations has not been reported previously, but a genotyping identification of *UGT1A1, SLCO1B1*, and *G6PD* variants in adult US residents found its occurrence. In one cohort study, nearly 50% of patients who carried the *G6PD A* – mutation coexpressed both homozygosity for either the A1463C or A388G *SLCO1B1* polymorphism and the *UGT1A1* $(TA)_7$ repeat promoter variant on ≥1 allele (Lin *et al*., 2008).

The individual contributions of *UGT1A1* and *SLCO1B1* variant expression to hyperbilirubinemia risk in infants who carry a *G6PD* mutation are not clear. Either *UGT1A1* or *SLCO1B1* variant suggests a synergistic effect (Watchko *et al*., 2009), compared with those who have *UGT1A1* or *SLCO1B1* polymorphisms only (Kaplan *et al*., 2007).

The statistical analysis of the study (Sato *et al*, 2015) revealed that *UGT1A1*, *SLCO1B1* and *SLCO1B3* polymorphisms become risk conditions in newborns showing 10% or more body weight loss during the neonatal period as maximal body weight loss was the only independent risk condition for the development of neonatal jaundice. Insufficient feeding can increase the bilirubin load and cause apparent jaundice in newborns, who have a polymorphic change in the genes participated in the bilirubin metabolism and/or transport.

2.2.5.4 Alpha-thalassemia gene

The thalassemia syndromes are the commonest monogenic conditions in human. They occur at an increase gene frequency throughout the Middle East, the Indian Subcontinent, the Mediterranean populations, and Myanmar, in a line stretching from southern China through Malay Peninsula and Thailand into the Pacific island populations. They are also found frequently in nations in which there has been migration from these high-frequency people. (Williams, 2016).

The pathophysiology is different in α-thalassemia because the excess β chains that consequence from defective α-chain production form hemoglobin H, β_4 molecules, which is soluble and does not precipitate in the bone marrow. Nevertheless, it is unstable and precipitates in older erythrocytes. Therefore, the anemia of α-thalassemia is due to hemolysis rather than dyserythropoietic (Williams, 2016).

2.2.5.4.1 Definitions and History

Cooley and Lee first defined a type of severe anemia that happened early in life in 1925 and was related with bone changes and splenomegaly. William L. Bradford and George H. Whipple published a comprehensive description of the pathologic observations in the disease in 1932. Whipple created the phrase *thalassic anemia* and shortened it to *thalassemia*, from θαλασσα ("the sea"), as primary patients were all of Mediterranean related (Williams, 2016).

2.2.5.4.2 Epidemiology and Population Genetics

The α-thalassemias happen broadly throughout the Southeast Asia, the Mediterranean countries, Africa, and Middle East. The α^0-thalassemias are found most frequently in Oriental and Mediterranean residents, but are tremendously rare in Middle Eastern and African peoples. Nonetheless, the deletion forms of α^+-thalassemia occur at an increase frequency through Southeast Asia, the Mediterranean, West Africa, and the Middle East (Williams, 2016).

2.2.5.4.3 Etiology and Pathogenesis

2.2.5.4.3.1 Genetic Control and Synthesis of Hemoglobin

Hemoglobin of human adult is a heterogenous combination of proteins comprising of the minor component hemoglobin A_2, which comprises approximately 2.5% of the total and the major component hemoglobin A. The major hemoglobin in intrauterine life is hemoglobin F. The structure of these hemoglobins is alike. Each hemoglobin composes of two discrete pairs of identical globin chains.

One pair of α-chains is present in all normal human hemoglobins excluding for some of the embryonic hemoglobins. The α-chains are joined with β chains ($\alpha_2\beta_2$) in hemoglobin A, in hemoglobin F with ɣ chains ($\alpha_2\gamma_2$), and in hemoglobin A_2 with δ chains ($\alpha_2\delta_2$) (Williams, 2016).

2.2.5.4.3.2 Globin Gene Cluster

Even though some subject variability exists, the α-gene cluster usually comprises two α genes, named α_1 and α_2 and one function ζ gene. It also comprises four pseudogenes: $\psi\alpha_1$, $\psi\alpha_2$, $\psi\zeta_1$, and θ_1. The latter is remarkably conserved between various species. Even though it seems to be expressed early in embryonal life, its function is unclear. The exons of the two α-globin genes have equal sequences. There is an identical sequence in the first intron in each gene. There are nine bases longer in the second intron of α_1 and varies by three bases from that in the α_2 gene (Williams, 2016).

Regulation of Globin Gene Clusters

The α-globin gene cluster also has a main regulatory element of this kind, in this case HS40. This forms part of four multispecies conserved sequences (MCSs), called MCS-R1-R4; or greatly conserved noncoding sequences, of these elements only MCS-R2 is HS 40, is vital for α-globin gene expression. Some of the thalassemia result from deletions are participating these regulatory regions. Furthermore, the phenotypic effects of deletions of these gene clusters are

powerfully positional, which may reflect the relative distance of particular genes from the HS40 and LCR (Williams, 2016).

2.2.5.4.4 α-Thalassemia Syndromes

The thalassemia syndromes are a heterogeneous group of hereditary diseases characterized by defects in the formation of one or more of the globin chain portions of the hemoglobin tetramer. The clinical features related with thalassemia arise from the integrated effects of imbalanced accumulation of globin subunits and inadequate hemoglobin production. The latter causes microcytosis and hypochromia; the former leads to hemolytic anemia and ineffective erythropoiesis. There are varied clinical presentations, ranging from asymptomatic hypochromia and microcytosis to profound anemia, which can be lethal in early childhood or in utero if not treated (Hoffman, 2012).

Alpha thalassemia syndromes are affecting 5% of world's people. The alpha globin gene is situated on chromosome 16p 13.3 forming (αα/αα) in a normal type. Absence of one alpha gene is known as a silent carrier (αα/α-), absence of two genes known as alpha thalassemia trait (-α/-α), (αα/--), absence of three genes known as HbH disease(--/-α) with inclusions in the erythrocytes stained with Brilliant Cresyl Blue are usually detected, and complete deletion of alpha genes known as Hb Barts hydrops fetalis (--/--), principally seen in Southeast Asia, is incompatible with life (Aliza *et al*., 2012).

Table 2.5: Classes of Mutations That Cause α-Thalassemia (Williams, 2016)

α^0-Thalassemia
Deletions downstream from α2 gene
Deletions involving both α-globin genes
Deletions of HS40 region
Truncations of telomeric region of 16p
α^+-Thalassemia
Deletions involving α_2 or α_1 genes
Point mutations involving α_2 or α_1 genes
mRNA processing
Splice site
Poly(A) signal
mRNA translation
Initiation
Nonsense, frameshift
Termination
Posttranslational
Unstable α-globin variants
α-Thalassemia Mental Retardation
ATR-16
Deletions or telomeric truncations of 16p

Translocation

ATR-X

Mutations of *ATR-X*

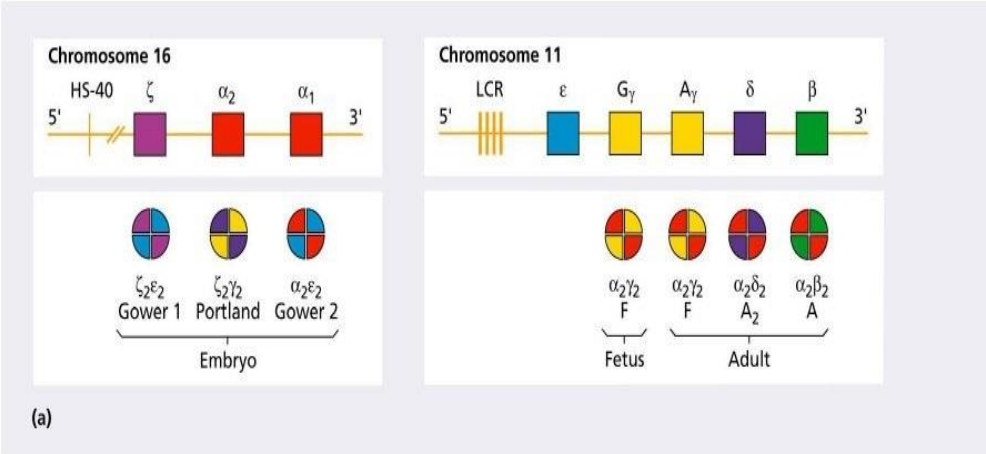
Deletions

Splice site

Missense

Nonsense

Table 2.5 summarizes the different types of α-thalassemia mutations. The α-globin gene haplotype can be mentioned αα, showing the α_2 and α_1 genes, respectively. A normal subject has the αα/αα genotype. In nondeletion thalassemia lesions, that is in which both genes haplotypes are intact, the nomenclature $\alpha^T\alpha$ is used, with the superscript T showing the gene is thalassemic. However, when the exact molecular defect is known, for instance, as in hemoglobin constant spring, $\alpha^T\alpha$ can be substituted by the more informative $\alpha^{CS}\alpha$. The population genetics and molecular pathology of the α-thalassemia syndromes have been the subject of numerous widespread reviews (Williams, 2016).



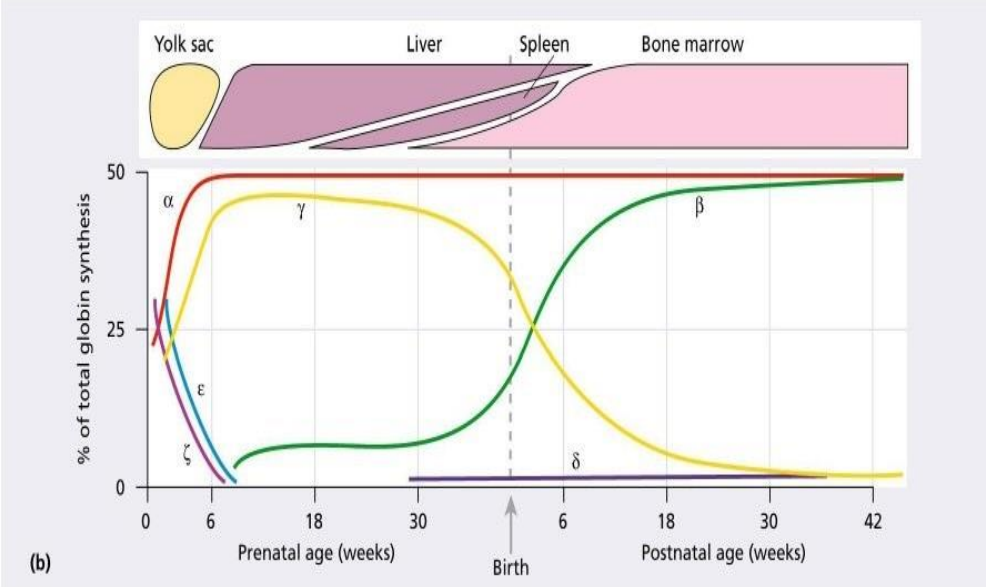


Figure 2.11:(a) The globin gene clusters on chromosomes 11 and 16. In embryonic, fetal and adult life different genes are activated or suppressed. The different globon chains are synthesized independently and then combine with each other to produce the different hemoglobins. The r gene may have two sequences, which code for either a glutamic acid or alanine residue at position 136 (G_r or A_r, respectively). LCR, locus control region. (b) Synthesis of individual globin chains in prenatal and postnatal life (Hoffbrand, 2015)

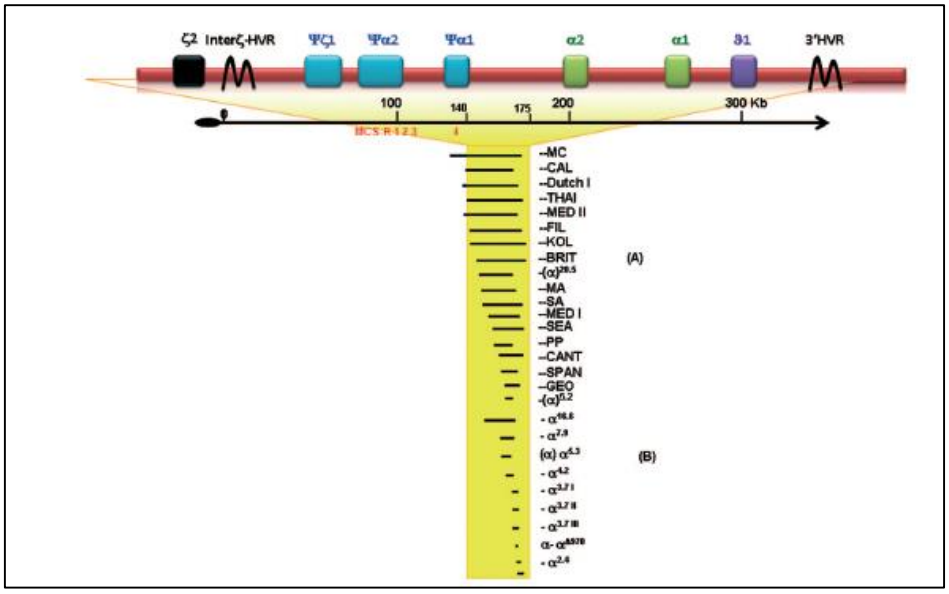


Figure 2.12: Alpha-globin gene clusters and deletions associated with α^0 (A) and α^+ (B) thalassemia. In normal individuals, alpha globin genes encoding the alpha globin chains are duplicated and localized in the telomeric region of chromosome 16 (16p 13.3), in a cluster containing also an embryonic zeta2 gene, encoding the embryonic zeta globin chains, three pseudogenes (pseudo zeta1, pseudo alpha1, and pseudo alpha2) and one gene (theta1) of unknown function (Galanello, 2011)

Etiology, Epidemiology, and Pathophysiology

The thalassemia syndromes are hereditary as pathologic alleles of one or more of the globin genes located on chromosomes 16 and 11. These conditions range from rearrangement of the loci to point mutations or total deletion that impair transcription, translation, or processing of globin mRNA. Thlassemias have been encountered in almost every geographic location and ethnic group. They are most commonly found in tropical or subtropical areas of Asia, Mediterranean and Africa. The "thalassemia belt" spreads along throughout the Arabian Peninsula, and the shores of the Mediterranean, Iran, Turkey, India, and Southeastern Asia, especially Thailand, southern China, and Cambodia (Hoffman, 2012).

2.2.5.4.5 Molecular Pathology and Pathophysiology

α^{0}- and α^{++}-thalassemia are denoted to as α-thalassemia-1 and α-thalassemia-2, respectively in the older literature. Nondeletional types of α-thalassemia constitute 15% to 20% of patients. The α-thalassemias have also associated with structurally abnormal hemoglobins. The α-globin chain Quong Sze ($\alpha^{125Leu\text{-}Pro}$) exists extremely unstable and is destroyed so quickly after its formation that no hemoglobin tetramers comprising the mutant α chain can be synthesized (Hoffman, 2012).

2.2.5.4.6 Clinical Presentations

Silent Carrier (α^{+}-Thalassemia Trait)

α^{+}-Thalassemia trait has no constant hematologic presentations. The erythrocytes are not microcytic, and Hb F and Hb A_2 are normal. Throughout the newborn period, small quantities (≤ 3%) of Hb Bart (γ_4) can be identified by other methods or electrophoresis (Hoffman, 2012).

2.2.5.4.7 Hb H Disease

Hb H disease is related with a moderately severe degree but variable anemia similar to thalassemia intermedia, with splenomegaly and bony changes. Nevertheless, the clinical phenotype may be noticeably milder in some individuals and severe enough to cause hydropsfetalis in others. It is rare in blacks but happens mostly in Asians and infrequently in whites (Mediterranean) (Hoffman, 2012).

2.2.5.4.8 Hydrops Fetalis with Hb Bart

Hemoglobin Bart Hydrops fetalis happens almost exclusively in Asians, especially Thais, Cambodians, Filipinos, and Chinese. Affected babies usually are born prematurely and either are died shortly after birth or stillborn. The erythrocytes are markedly hypochromic and microcytic and comprise large numbers of circulating nucleated red blood cells and target cells (Hoffman, 2012).

2.2.5.4.9 Nondeletion α-Thalassemia

Since the α2 gene expression is two or three times greater than the α1 gene expression, the result that majority of the nondeletion mutants detected to date affect mainly α2 gene expression is not astonishing. Probably this is ascertainment bias for the reason that of the greater phenotypic consequence of these conditions. It is also likely that the α2 gene defective expression has come under greater selective pressure (Williams, 2016).

2.2.5.4.10 Interactions of α-thalassemia Haplotypes

Various α-thalassemia haplotypes have been identified, and potentially more than 500 interactions are likely. Phenotypically, these phenotypes consequence in four broad categories: normal, situations characterized by mild hematologic changes but no clinical abnormality, hemoglobin H disease, and hemoglobin Bart's hydrops fetalis syndrome (Williams, 2016).

2.2.5.4.11 Unusual Forms of α-Thalassemia

Some α-thalassemia unusual forms are entirely unrelated to the common forms of the disease that happen in tropical regions. These situations, which can happen in any racial groups, consists of α-thalassemia related with leukemia or mental retardation. Their significant lies with the diagnostic difficulties they may present and, more significantly, the light that clarification of the α-thalassemia pathology may shed on wider disease (Williams, 2016).

2.2.5.4.12 Thalassemia as a Global Health Problem

The outstanding advances in the diagnosis, management, and prevention of the thalassemia syndromes are only the appropriate to the wealthier nations of the world. In several developing nations in which there is a very high occurrence of thalassemia, there are very inadequate facilities for their treatment and diagnosis. As many of these nations are going through the epidemiologic transition, which includes cleaner water supplies, improvements in nutrition, and well public health services, patients with serious types of thalassemia who previously would have died of profound anemia or infection are now surviving to present for therapy (Williams, 2016).

Alpha thalassemia is common in Malaysia affecting the Malays, Chinese, Indians and other ethnics. Aliza *et al* reported that the most common mutation in the Malays was $-\alpha^{3.7}$ and $--^{SEA}$ in the Malaysian-Chinese. In this study the $--^{SEA}$, $-\alpha^{3.7}$ and $-\alpha^{4.2}$ were observed with different frequencies in Malays and Malaysian-

Chinese. This study also stated that $--^{SEA}$ was seen in 1.1% Malay and 5.4% of Chinese. The $-\alpha^{3.7}$ was seen in 1.6% Chinese and 4.3% Malay. $-\alpha^{4.2}$ was seen in 0.9% Chinese. The present prevalence and projected number of pregnancies at risk of deletional HbH disease and deletional Hb Barts hydrops fetalis syndrome will help to attain a better disease treatment in order to decrease present alpha thalassemia burden and to prevent much higher alpha thalassemia birth in Malaysia (Aliza *et al*., 2012).

Previous to 2004, the country had no national strategy for registry and screening for thalassemia. Due to lacking of national audit, the accurate figure of the degree of thalassemia in the Malaysian people was mostly presumptive from micro-mapping studies from numerous researchers in the nation (George and Mary, 2011).

There have been studied on the prevalence of *G6PD* deficiency in thalassemia adult individuals (Bernaudin *et al*., 2008). Tan *et al* study reported that the most frequently mutations occurring of α-thalassemia are the $-\alpha^{4.2}$ and $-\alpha^{3.7}$ single α-globin gene deletions, while double α-globin gene deletions in *cis*, such as the $--^{SEA}$, $--^{THAI}$, and $--^{FIL}$ alleles are very common in Southeast Asia, and the $-(\alpha)^{20.5}$ and $--^{MED}$ double-gene deletions occur more frequently in the Mediterranean area (Tan *et al*., 2001).

Mary Anne Tan *et al* study reported that alpha-thalassemia was observed in the Malaysia Kadazandusuns as the single α-globin gene deletion ($-\alpha^{3.7}$) was confirmed in 33.6% of the subjects (Mary Anne Tan *et al*., 2010). The earlier study (Wee *et al*., 2005) described that only 10.7% of Malaysian Malays, 10.0% of Chinese, and 7.4% of Indian possessed this deletion.

Aliza *et al*., 2012 stated that 17 (7.4%) of blood donor subjects in Malaysia showed alpha thalassemia mutations. Of which, 3.5% were with double gene deletions and 3.9% with single gene deletions. Rosnah *et al* study reported that among the 400 first-time blood donors studied in Malaysia, 37 of them (9.3%) were found to have α-thalassemia of deletion type. 34 subjects (8.0%) were heterozygous for $\alpha^{3.7}$ mutation while 1 subject (0.3%) was showed to have heterozygous $\alpha^{4.2}$ deletion, and 2 subjects (0.5%) had heterozygous SEA-type deletion (Rosnah *et al*., 2012).

Nainggolan *et al* study observed that the α-thal subjects who had Hb Adana in combination with the 3.7 kb deletion mostly have mild-to-moderate degree of anemia in Indonesia. In contrast, patients who were compound heterozygotes for nondeletional mutations and Hb Adana, generally exhibited a more severe anemia and it mostly found in childhood. Their study also reported that eighteen cases (67.0%) were compound heterozygotes for Hb Adana and either the $-\alpha^{3.7}$ (17 cases) or $-\alpha^{4.2}$ (one case) mutations, and nine cases (33.0%) were compound heterozygotes for Hb Adana and other nondeletional mutations (Nainggolan *et al*., 2013).

Hemoglobin CS is the most common α-globin structural variant in Malaysia and in other Southeast Asian countries and it ranges between 1-8% (Lie-Injo and Duraisamy, 1972, Laig *et al*., 1990 Jomoui *et al*., 2015). There were few young adult or adult case reports on mutation of Hb CS and Hb Adana in Malaysia (Lie-Injo, 1974, George, 2009, Wee, 2009, Azma, 2012), and these reports stated that Hb CS (HbA2: c.427T>C) and hemoglobin Adana (HbA2: c.179G>A) mutations were associated with jaundice.

Factors identified to be related with non-physiologic neonatal hyperbilirubinemia include low birth weight, prematurity, East Asian ancestry, breast-milk feeding and a sibling with neonatal jaundice. However, no known aetiologies were observed in nearly half of these neonatal hyperbilirubinemia cases (Wong, 2013). Gene variant may play an important role in the development of neonatal hyperbilirubinemia (Weng, 2016). Although the exact aetiologies of neonatal jaundice has not been fully understood, perinatal and genetic factors are widely accepted to jointly contribute to this disease (Zhou, 2018).

CHAPTER 3

MATERIALS AND METHODS

This study was a descriptive study carried out in newborns admitted over an eighteen-month period in the Selayang Hospital.

Inclusion criteria

- Term infants (gestation ≥ 37 weeks)
- Normal born and well infants (Apgar score of 8 and above at 1 and 5 minutes, and asymptomatic after birth) who developed jaundice after being discharged from hospital with their mothers

Exclusion criteria

- Term infants who were born unwell with medical illness (other than mild jaundice) needed intensive care treatment after birth
- Major congenital malformations
- Preterm infants of gestation less than 37 completed weeks
- No written parental consent

The sampling method in the present study was ‘Universal Sampling’ as we recruited all hospitalized jaundiced neonates who provided consent and were

eligible following the inclusion criteria. Examination and determination of inclusion and inclusion criteria were done by the specialist pediatricians according to the standard of care in hospital and guidelines. Serial measurement of TSB level was done in every infant and from which the highest level of TSB was used for the classification of bilirubin levels. A written consent was obtained from the mother or father of all eligible infants for participation in this study. Total serum bilirubin level of ≥291 umol/L (≥17 mg/dL) was considered as SigNH whereas newborns admitted with TSB <291 umol/L (<17mg/dL) were considered as non-SigNH.

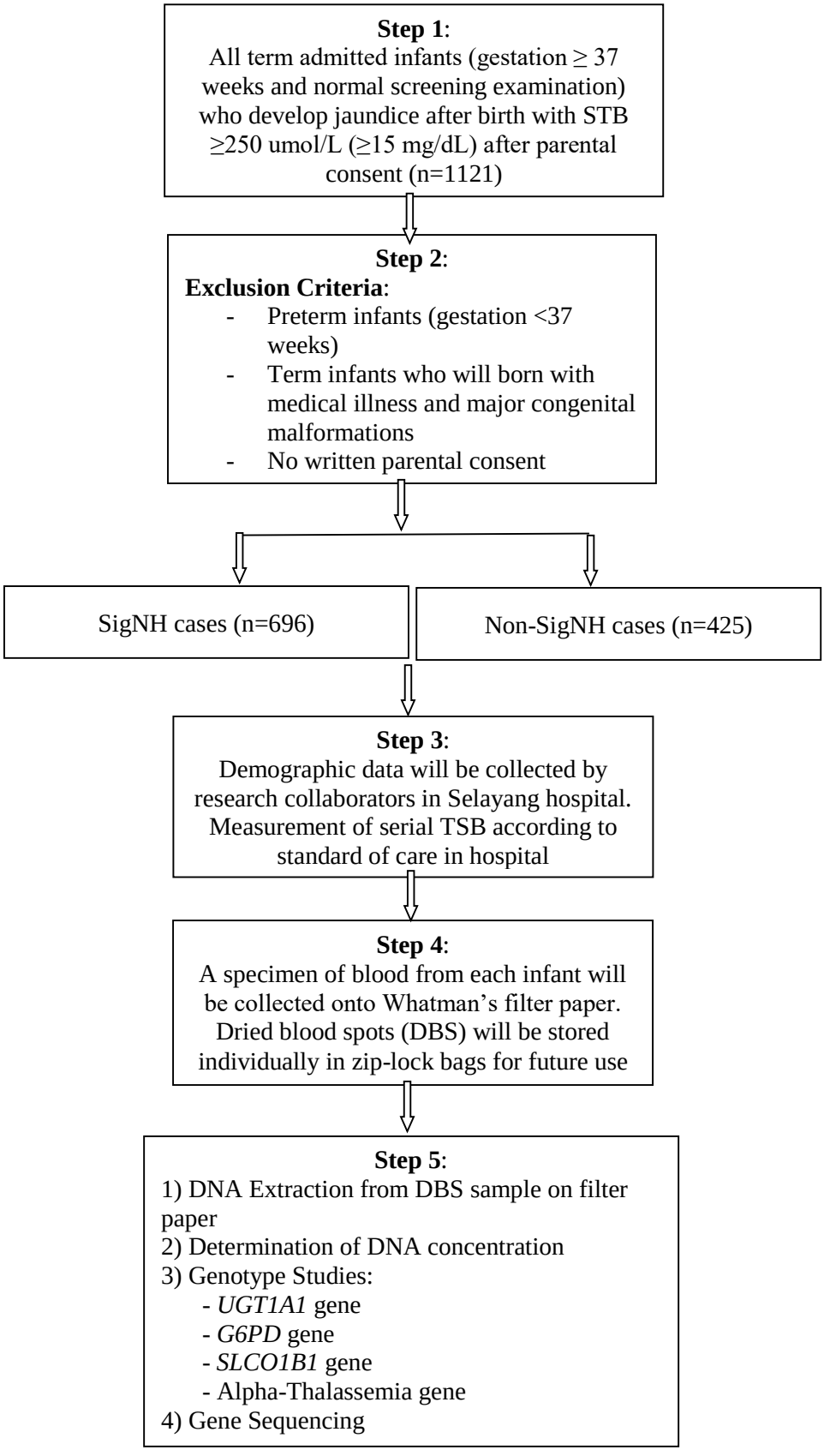
Sample size calculation

Based on the findings of previous study on Malaysian infants (Ainoon, 2002), a minimum sample of at least 243 infants with severe jaundice and 243 infants without severe jaundice need to be recruited in order to detect a significant predictor with an anticipated odds ratio of 5.0 with a relative precision of 50% with 90% level of confidence. In this study, we recruited 1121 hyperbilirubinemic neonates with TSB ≥250 umol/L (≥15 mg/dL) according to the selection criteria and there were 696 SigNH and 425 non-SigNH cases participated.

Ethnicity

This study recruited three Malaysian ethnic groups (Malay, Chinese and Indians) and others (mixed ethnicity).

3.1 Research Flow



3.2 Sample Storage

The specimens of blood from each infant were collected onto Whatman's filter paper (Bioline, USA), dried blood spots (DBS) were kept separately in zip-lock bags containing silica desiccant beads (MERCK, Germany) and transported to the research laboratory in UTAR for DNA analysis.

3.3 DNA Extraction from DBS samples

Genomic DNA Extraction and Purification from sample on FTA Elute card (Bioline, USA)

1.2 mm disc (10 punches) were removed from the dried sample by using a Harris punch tool and placed into a 1.5 ml microcentrifuge tube. Then, 500 ul of sterile water was added and vortexed 5 times. FTA Elude chemicals and cellular debris were washed from the disc while proteins remained bound to the disc. The rinse water was centrifuged briefly and removed. The washed discs were transferred to a clean 0.5 ml microcentrifuge tube by using a pipette tip.

30ul of sterile water was added and incubated in a thermal cycler or calibrated heating block at 95ºC for 30 min. After incubating the disc, vortex for 1 min by 60 times pulsing the tubes to dislodge the DNA from the matrix. Throughout heating, DNA was dissociated and denatured from the fibers of the FTA card. Proteins and other PCR inhibitors continued bound to the matrix. The tube was centrifuged to recover the condensation from the top of the tube and to pellet the disc. The latter was then detached from the solution and the eluted DNA was stored.

3.4 Determination of DNA concentration

Concentration of DNA and estimation of purity were determined by using Nano Photometer (IMPLAN, Germany) at an absorbance (A) of 260 nm and 280 nm.

3.5 Genotypes studies

3.5.1 *UGT1A1* Genotype Study

PCR-Restriction Fragment Length Polymorphism Analysis (PCR-RFLP)

PCR-restriction fragment length polymorphism (RFLP) method was used to detect the known variant sites in the *UGT1A1* gene namely promoter area, nucleotide 211, c.-3279, 686, 1091 and 1456. The mutagenic or natural primers, restriction enzymes, and digested restriction fragment sizes of the six known variants are listed in Table 3.1. The PCR mixture (25 ul) consisted of 200 ng of DNA, 1 ul of each primer (20 uM each), 12.5 ul of Mytaq Mix (BiolineInc, U.S.A) and 8.5 ul of water (ddH_2O). PCR amplification was performed in a DNA thermal cycler (applied Biosystems, Veriti) for 35 cycles of initial denaturation for 1 min at 95°C, annealing for 15 sec at 55-59°C and primer extension for 10 sec at 72°C. The PCR product was digested with the appropriate restriction enzyme and analyzed on a 3% agarose gel (NHK Bioscience Solutions Sdn Bhd). All PCR reagents, primers and PCR products were stored at -20˚C.

3.5.1.1 Optimization

After developing a procedure for PCR amplification of a target variant, it was important to optimize all parameters comprising cycling temperatures, cycle number, and reagent concentrations. We foremost calculated the volume of each reagent required in a PCR reaction and kept the primer concentration constant. After completed the calculations, we prepared a master mix, a solution containing all the components for PCR amplification with the exception of template. Once master mix was prepared, aliquot of master mix solution dispersed equally into 7 reaction tubes and DNA template was added as the last component to each of the 6 tubes.

The 7th tube was added water in order to prepare non-template negative control tube. The final volume of each reaction was 50 ul. To optimize the annealing condition, we applied gradient temperatures in thermal cycler and noted down as an optimized temperature for the most prominent band product of the desired size.

Table 3.1: Mutagenesis or natural primers, restriction enzymes, and *UGT1A1* gene variations (Huang, 2004, Huang, 2008)

Position (cDNA)	Primers	Sequence	Restriction enzyme	Result (bp)
211 G>A	U1F1	5′AGA TAC TGT TGA TCC CAG TG3′	*Ava II*	G 128 + 18
	U211R	5′CTT CAA GGT GTA AAA TGG TC3′		A 146
686 C>A	U1F4	5′CAC TGT ATT CTT CTT GCA TG3′	*Bsr I*	C 374 + 51
	U1R2	5′CGA TCC AAA GTA ATA CAT CTG3′		A 242 + 132 + 51
1091 C>T	U4F2	5′GCC AAC ATA TCC TAC ATT GC3′	*Bcl I*	C 209
	U1091R	5′GTG ATA AAG GCA CGG GTG AT3′		T 190 + 19
1456 T>G	U5F3	5′GTG GAG TTT GTG ATG AGG CA3′	*Ava II*	T 270
	U5R1	5′GGA AAT GAC TAGGGA ATG GT3′		G 197 + 73
Promoter	(TA)F	5′TAA CTT GGT GTA TCG ATT GGT3′		$A(TA)_6TAA$ 77
$A(TA)_nTAA$	(TA)R	5′CTT TGC TCC TGC CAG AGG TT3′		$A(TA)_7TAA$ 79
3279 T>G	3279F	5′CAC CAG AAC AAA CTT CTG3′	*Dra I*	T 121+20
	3279R	5′GTT CTC AAA TTG CTT TGT TTA3′		G 141

Table 3.2: PCR mixture for amplification with Standard MyTaq Protocol for *UGT1A1* gene variations (Bioline, USA)

Template	200 ng
Primers (20 uM each)	1 ul
MyTaq Mix, 2x	25 ul
Water (ddH_2O)	Up to 50 ul

Table 3.3: PCR cycling conditions for *UGT1A1* gene variations (Bioline, USA) (Huang, 2004, Huang, 2008)

Step	Temperature	Time	Cycles
Initial denaturation	95C	1 min	1
Denaturation	95C	15 sec	33-35
Annealing	55-59C	15 sec	
Extension	72C	10 sec	

Table 3.4: Restriction enzyme reaction condition *UGT1A1* gene variations (New England Biolab, UK Protocol)

Restriction Enzyme	1 ul
DNA	1 ul
10x NEBuffer	5 ul (1x)
Total Rxn Volume	50 ul
Incubation Temperature	As mentioned in protocol
Incubation Time	5-15 min

3.5.2 *G6PD* Genotype Study

3.5.2.1 PCR-Restriction Fragment Length Polymorphism Analysis (PCR-RFLP)

PCR-restriction fragment length polymorphism (RFLP) method was applied to detect the known variant sites in the *G6PD* gene namely Mediterranean, Canton, Kaiping, Mahidol, Mediterranean, Vanua Lava, Chatham, and Orissa. The natural or mutagenic primers, restriction enzymes, and digested restriction fragment sizes of these variants are listed in Table 3.5. The PCR mixture (25 ul) consisted of 200 ng of DNA, 1 ul of each primer (20 uM each), 12.5 ul of Mytaq Mix (BiolineInc, U.S.A) and 8.5 ul of water (ddH_2O). PCR amplification was performed in a DNA thermal cycler (applied Biosystems, Veriti) for 35 cycles of initial denaturation for 3 min at 95°C, annealing for 15 sec at 55-67°C and primer extension for 60-90 sec at 72°C. The PCR product was digested with the appropriate restriction enzyme and analyzed on a 3% agarose gel (NHK Bioscience Solutions Sdn Bhd). All PCR reagents, primers and PCR products were stored at -20˚C.

Table 3.5: Natural or mutagenesis primers, restriction enzymes, and *G6PD* gene variations (Ainoon, 2002, Ainoon, 2004)

Position (cDNA)	Primers	Sequence	Restriction enzyme	Result (bp)
nt 1388 G>A	1388F 1388R	5′ACG TGA AGC TCC CTG ACG C3′ 5′GTG CAG CAG TGG GGT GAA CAT A3′	*Nde I*	G 227 A 205+22
nt 871 G>A	871F 871R	5′TGG CTT TCT CTC AGG TCT AG3′ 5′GTC GTC CAG GTA CCC TTT GGG G3′	*Xba I*	G 126 A 106+20
nt 1376 G>T	1376F 1376R	5′ACG TGA AGC TCC TGA CGC3′ 5′TGA AAA TAC GCC AGG CCT CG3′	*Xho I*	G 213 T 192+21
nt 563 C>T	563F 563R	5′GGT GAG GCT CCT GAG TAC CA3′ 5′AGC TGT GAT CCT CAC TCC CC3′	*Mbo II*	C 377+119+26+25 T 277+119+100+26+25
nt 487 G>A	487F 487R	5′GCG TCT GAA TGA TGC TGC TGT GAT3′ 5′AGC CGG TCA GTG CTC TGC ATG TCC3′	*Alu I*	G 82+60 A 62+60+20
nt 383 T>C	383F 383R	5′CAA CAG CCA CAT GGA TGA CCC3′ 5′AGG CGG GAA GGG AGG GC3′	*Mnl I*	T 121+20 G 141
nt 1003 G>A	1003F 1003R	5′CAA GGA GCC CAT TCT CTC CCT T3′ 5′TTC TCC ACA TAG AGG ACG ACG GCT GCC AAA GT3′	*Bst XI*	G 208 A 130+78
nt 131 C>G	131F 131R	5′CAG CCA CTT CTA ACC ACA CAC CT3′ 5′CCG AAG TTG GCC ATG CTG GG3′	*Hae III*	C 107+75+66+48+45+11 G 123+107+66+45+11

Table 3.6: PCR mixture for amplification with Standard MyTaq Protocol for *G6PD* Gene variations (Bioline, USA)

Template	200 ng
Primers (20 uM each)	1 ul
MyTaq Mix, 2x	25 ul
Water (ddH_2O)	Up to 50 ul

Table 3.7: PCR cycling conditions for *G6PD* Gene variations (Bioline, USA) (Ainoon, 2002, Ainoon, 2004)

Step	Temperature	Time	Cycles
Initial denaturation	95C	3 min	1
Denaturation	95C	30 sec	35
Annealing	55-67C	15 sec	
Extension	72C	60-90 s	

Table 3.8: Restriction enzyme reaction condition for G6PD Gene variations (New England Biolab, UK Protocol)

Restriction Enzyme	1 ul
DNA	1 ul
10x NEBuffer	5 ul (1x)
Total Rxn Volume	50 ul
Incubation Temperature	As mentioned in protocol
Incubation Time	5-15 min

3.5.3 *SLCO1B1* Genotype Study

3.5.3.1 PCR-Restriction Fragment Length Polymorphism Analysis (PCR-RFLP

PCR-restriction fragment length polymorphism (RFLP) method was applied to detect the known variant sites in the *SLCO1B1* gene namely 388G>A and 521 T>C. The natural or mutagenic primers, restriction enzymes, and digested restriction fragment sizes of these variants are listed in Table 3.9. The PCR mixture (25 ul) consisted of 200 ng of DNA, 1 ul of each primer (20 uM each), 12.5 ul of Mytaq Mix (Bioline Inc, U.S.A) and 8.5 ul of water (ddH_2O). PCR amplification was performed in a DNA thermal cycler (applied Biosystems, Veriti) for 35 cycles of initial denaturation for 3 min at 95°C, annealing for 15 sec at 48°C and primer extension for 10 sec at 72°C. The PCR product was digested with the appropriate restriction enzyme and analyzed on a 3% agarose gel (NHK Bioscience Solutions Sdn Bhd). All PCR reagents, primers and PCR products were stored at -20˚C.

Table 3.9: Natural or mutagenesis primers, restriction enzymes, and *SLCO1B1* gene variations (Huang, 2004)

Position (cDNA)	Primers	Sequence	Restriction enzyme	Result (bp)
388G>A	388F	5′ATA ATG GTG CAA ATA AAG GGG3′	*Taq I*	G 128+63+23
	388R	5′ACT ATC TCA GGT GAT GCT CTA3′		A 151+63
521T>C	521F	5′TTG TCA AAG TTT GCA AAG TG3′	*Hha I*	T 209
	521R	5′GAA GCA TAT TAC CCA TGA GC3′		C 189+20

Table 3.10: PCR mixture for amplification with Standard MyTaq Protocol for *SLCO1B1* Gene variations (Bioline, USA)

Template	200 ng
Primers (20 uM each)	1 ul
MyTaq Mix, 2x	25 ul
Water (ddH_2O)	Up to 50 ul

Table 3.11: PCR cycling conditions for *SLCO1B1* Gene variations (Bioline, USA) (Huang, 2004)

Step	Temperature	Time	Cycles
Initial denaturation	95C	3 min	1
Denaturation	95C	15 sec	33-35
Annealing	48C	15 sec	
Extension	72C	10 s	

Table 3.12: Restriction enzyme reaction condition for S*LCO1B1* Gene variations (New England Biolab, UK Protocol)

Restriction Enzyme	1 ul
DNA	1 ul
10x NEBuffer	5 ul (1x)
Total Rxn Volume	50 ul
Incubation Temperature	As mentioned in protocol
Incubation Time	5-15 min

3.5.4 Alpha-Thalassemia Genotype Study

3.5.4.1 PCR-Restriction Fragment Length Polymorphism Analysis (PCR-RFLP)

PCR-restriction fragment length polymorphism (RFLP) method was applied to detect the known variant sites in the non-deletional alpha-thalassemia gene namely Hb Constant Spring (CS) and Hb Adana. The natural or mutagenic primers, restriction enzymes, and digested restriction fragment sizes of these variants are listed in Table 3.13. The PCR mixture (25 ul) consisted of 200 ng of DNA, 1 ul of each primer (20 uM each), 12.5 ul of Mytaq Mix (Bioline Inc, U.S.A) and 8.5 ul of water (ddH_2O). PCR amplification was performed in a DNA thermal cycler (applied Biosystems, Veriti) for 35 cycles of initial denaturation for 5 min at 95°C, annealing for 15-30 sec at 62-66°C and primer extension for 90 sec at 72°C. The PCR product was digested with the appropriate restriction enzyme and analyzed on a 3% agarose gel (NHK Bioscience Solutions Sdn Bhd). All PCR reagents, primers and PCR products were stored at -20˚C.

Table 3.13: Natural or mutagenesis primers, restriction enzymes, and Hb CS and Hb Adana gene variations (Nainggolan, 2013)

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

Table 3.14: PCR mixture for amplification with Standard MyTaq Protocol for non-deletional alpha-thalassemia gene variations (Bioline, USA)

Template	200 ng
Primers (20 uM each)	1 ul
MyTaq Mix, 2x	25 ul
Water (ddH_2O)	Up to 50 ul

Table 3.15: PCR cycling conditions for non-deletional alpha-thalassemia gene variations (Bioline, USA) (Nainggolan, 2013)

Step	Temperature	Time	Cycles
Initial denaturation	95C	5 min	1
Denaturation	95C	15-30 sec	35
Annealing	62-66C	15 sec	
Extension	72C	90 s	

Table 3.16: Restriction enzyme reaction condition for non-deletional alpha-thalassemia gene variations (New England Biolab, UK Protocol)

Restriction Enzyme	1 ul
DNA	1 ul
10x NEBuffer	5 ul (1x)
Total Rxn Volume	50 ul
Incubation Temperature	As mentioned in protocol
Incubation Time	5-15 min

3.5.5 Electrophoretic separation and visualization of fragments

After accomplishment of digestion of amplicons with the selected restriction enzyme(s), the resultant fragments were determined by electrophoresis. This was done using agarose as molecular sieving matrix with slab gel electrophoresis.

Agarose gel electrophoresis

Reagents for gel electrophoresis

1. NuSieve R 3:1 Agarose powder (NHK Bioscience)
2. X10 TBE Solution (Axil Scientific, Singapore)
3. RedSafe Nucleic Acid Staining Reagent (INTRON Biotechnology, Korea)
4. 6x loading dye (INTRON Biotechnology, Korea)
5. DNA ladder/ marker (100-1000 base pairs) (Axon Scientific, Malaysia)

Preparation of TE-saturated phenol

The phenol condition was critical to the quality of the DNA obtained. 500 ml of water was taken and prepared 500 ml 0.5 mol/L Tris pH 8.5, and mixed by inversion for 2-3 min. The mixture was left to stand until the organic and aqueous phase have separated. Then the upper aqueous layer was removed and discarded. Then another 125 ml of 0.5 mol/L Tris was added, stood, take out the aqueous layer as before, and then repeated. The residual 100 ml of 0.5 mol/L Tris was added 900 ml of water to get 500 ml of 0.1 mol/L Tris. Then 150 ml of 0.1 mol/L Tris was added to the phenol and then mixed, stood, and removed the upper aqueous layer

and repeated two more time. The residual 50 ml of 0.1 mol/L Tris was adding 449 ml of water and 1 ml of 0.5 mol/L EDTA to provide 500 ml of TE. This included three stages as earlier. The phenol has decreased in volume during this process.

X10 TBE buffer (Tris-Borate EDTA)

Trisma base	18.15 gm
Boric acid	9 gm
EDTA.3 Na	1.23 gm
Double distilled ionized water	300 ml

3.5.5.1 Method of agarose gel electrophoresis

Preparation of 3% agarose gel

Agarose powder 9gm was measured and added it to a 500ml flask. Then TBE buffer 300ml was added to the flask (total gel volume would be different depending on the size of the casting tray). The agarose was melted in a microwave oven until the solution developed was clear. The solution was heated for numerous short intervals. Then the solution was cooled to about 45-55˚C, the flask was swirled infrequently to evenly cool. The combs were engaged in the gel casting tray. Then 2 ul of Gel-red solution (RedSafe Nucleic Acid Staining Solution 20,000x, INTRON, Korea) was added to melted solution. The liquefied agarose solution was transferred into the casting tray and let it cool until it was solid (it should appear as milky white). The combs were carefully removed and placed the gel in electrophoresis chamber. TBE buffer was poured enough so that there was about 3-

4 mm of buffer over the gel. Gel could be prepared some days earlier to use and wrapped in plastic wrap without combs.

Loading the gel

The order of each sample was recorded and 2ul of 6X sample loading buffer was added to each 10ul PCR products. 12ul of each sample loading buffer mixture was carefully loaded into isolated wells in the gel and 4ul of the DNA ladder was pipette standard into at minimum one well of each row on the gel.

Running the gel

The lid was placed on the gel box and the electrodes were connected. Then the electrode wires were connected to the power supply, making sure the positive (red), negative (black) were correctly connected. The power supply was turned on to about 100 volts. To make sure that the current was running in the correct direction by observing the movement of the blue loading dye. This would take a couple of minutes (it would run in the same direction as the DNA). The power was allowed to run until the blue dye approaches the end of the gel and approximately it took 40 minutes. The power was turned off and the wires disconnected from the power supply. The lid of the electrophoresis chamber was removed then the tray and gel were carefully removed.

Gel staining & documentation

Finally, DNA as well as PCR products were visualized under UV trans-illuminator to identify the presence or absence of products and determine the size of the amplified fragemnts. Images were captured with UVP BioSpectrum Imaging System (BioSpectrum, UK).

3.6 Gene Sequencing

PCR products of all positive mutations were sent for Gene sequencing for verification. All the findings were matched with those determined by the sequencing method (*Applied Biosystems 3730XL* DNA Analyzer and Applied Biosystems Sequence Scanner Software for sequence result analysis, U.S.A) Sequence data was compared to 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/.

3.7 Statistical analysis

Statistical analysis was conducted by using IBM SPSS Statistic 22.0 for Windows software program 2013 (SPSS Inc., Chicago, IL, USA). Data were expressed as percentage for categorical variables. Fisher's exact test was used for comparisons of frequencies of *UGT1A1, G6PD, SLCO1B1* and alpha-thalassemia variants between male and female newborn babies, between the major ethnic groups, and between SigNH and non-SigNH babies. Logistic regression, odds ratio

and 95% confidence interval were used for identification of risk factors. A P-value of <0.05 was considered significant.

CHAPTER 4

DEMOGRAPHIC DATA AND URIDINEDIPHOSPHATE-GLUCURONOSYLTRANSFERASE 1A1 (*UGT1A1*) GENE POLYMORPHISMS

4.1 Results

In this study, we recruited 1121 hyperbilirubinemic neonates with TSB ≥250 umol/L (≥15 mg/dL) according to the selection criteria. Out of a total 1121 subjects, SigNH and non-SigNH were 696 cases and 425 cases respectively. The three ethnic groups and gender distribution of hyperbilirubinemia are shown in the Figure 4.1, Figure 4.2 and Table 4.1. Majority were Malays (74.9%, 839 of 1121) compared to Malaysian Chinese (16.9%, 190 of 1121), Malaysian Indian (3.2%, 36 of 1121) and Others (5.0%, 56 of 1121). Males had 51.2% (574 of 1121) and females showed 48.8% (547 of 1121) in this study.

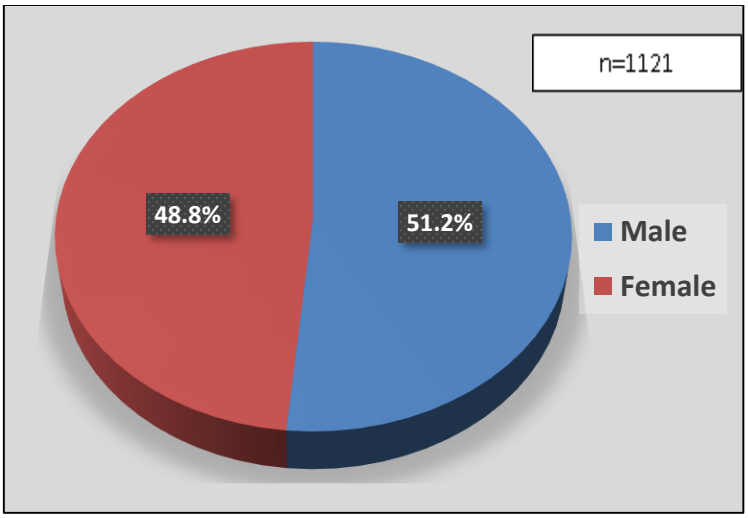


Figure 4.1: Gender distribution of the study

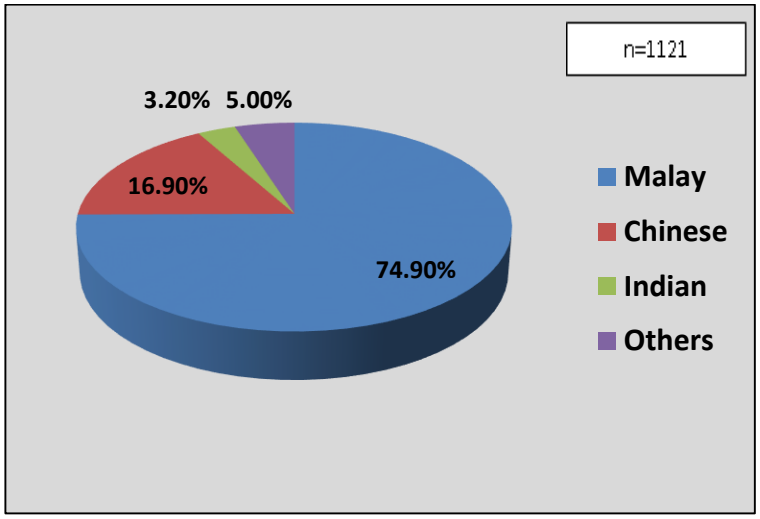


Figure 4.2: Ethnic groups of neonates showing Malay, Chinese, Indian and others

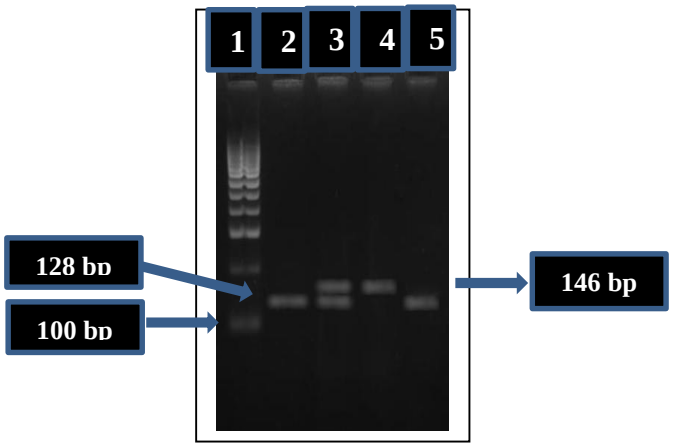
Table 4.1: Demographic characteristics

Category	*n = 1121*		OR (95% CI)	p-value
	SigNH (TSB≥291 umol/L) (*n* = 696)	**Non-SigNH (TSB<291 umol/L) (*n* = 425)**		
Gender				
Male	362 (63.1%)	212 (36.9%)	1.09 (0.86-1.39)	0.49
Female	334 (61.1%)	213 (38.9%)		
Races				
Malay (n=839)	515 (61.4%)	324 (38.6%)	1.27 (0.91-1.77)	0.34
Chinese (n=190)	127 (66.8%)	63 (33.2%)	1.80 (0.88-3.71)	0.34
Indian (n=36)	19 (52.8%)	17 (47.2%)	1.42 (0.73-2.78)	0.81
Others (n=56)	35 (62.5%)	21 (37.5%)	1.05 (0.60-1.83)	0.33

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

Out of total 1121 subjects studied, the result of the present study showed that 70.7% (793/1121) of the main ethnic groups of neonates carried a *UGT1A1* gene mutation.



Lane 1: Ladder 100 bp
Lane 2 and 5: GG homozygote
Lane 3: GA heterozygote
Lane 4: AA homozygote

Figure 4.3: PCR and restriction pattern of (211G>A) *UGT1A1* gene. Lane 1 is a 100 bp DNA marker. Lane 2 to 5 are restriction fragment pattern of PCR products after digesting with *Ava II* and running in 3% agarose gel

In Figure 4.3, the primary PCR product for 211G>A was 146 bp long and included two *Ava II* sites which resulted in three restriction fragments of 146 bp, 128 bp, and 18 bp. Homozygous AA genotype was represented by one fragment (146 bp) meanwhile homozygous GG genotype was represented by two fragments (128 bp and 18 bp). PCR products of heterozygote GA was digested at two restrictions sites and produced three fragments (146 bp, 128 bp and 18 bp). However, fragment of 18 bp could not be observed clearly due to small fragment size.

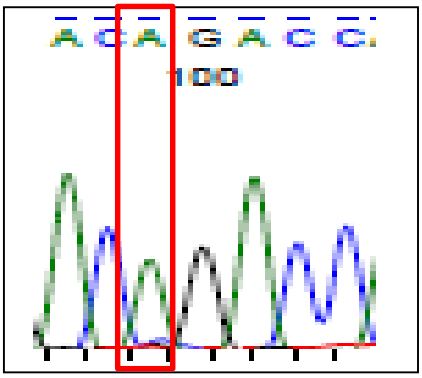
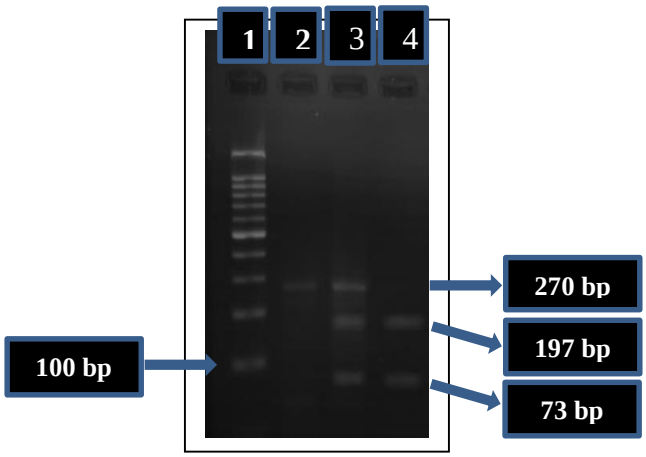


Figure 4.4: Sequence chromatogram of *UGT1A1* gene variant 211G>A showing AA homozygous mutation

Figure 4.4 shows the sequence chromatogram of *UGT1A1* gene variant homozygous 211G>A. The 211G>A variant showed 2 heterozygous variations (66.7%) and 20 homozygous variations (90.9%) in SigNH group and 1 case of heterozygous (33.3%) and 2 cases of homozygous (9.1%) in non-SigNH.



Lane 1: Ladder 100 bp
Lane 2: TT homozygote
Lane 3: TG heterozygote
Lane 4: GG homozygote

Figure 4.5: PCR and restriction pattern of (1456T>G) *UGT1A1* gene. Lane 1 is a 100 bp DNA marker. Lane 2 to 4 are rrestriction fragment pattern of PCR products after digesting with *Ava II* and running in 3% agarose gel

In Figure 4.5, the primary PCR product for 1456T>G was 270 bp long and included two *Ava II* sites which resulted in three restriction fragments of 270 bp, 197 bp, and 73 bp. Homozygous TT genotype was represented by one fragment (270 bp) meanwhile homozygous GG genotype was represented by two fragments (197 bp and 73 bp). PCR products of heterozygote TG was digested at two restrictions sites and produced three fragments (270 bp, 197 bp and 73 bp).

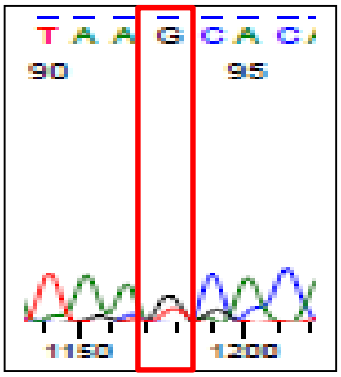
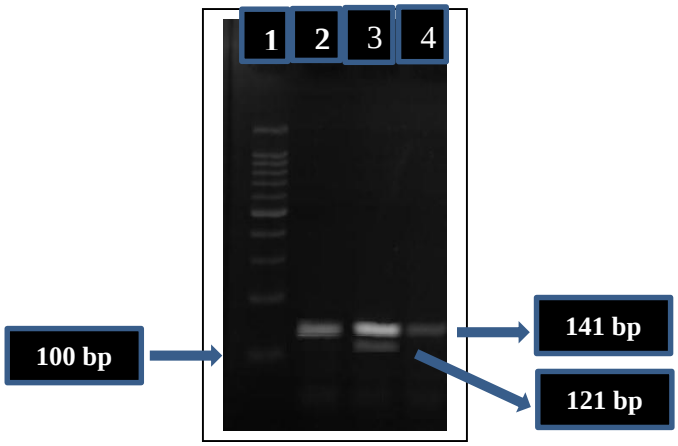


Figure 4.6: Sequence chromatogram of *UGT1A1* gene variant 1456T>G showing TG heterozygous mutation

Figure 4.6 shows sequence chromatogram of *UGT1A1* gene heterozygous variant 1456T>G. The variant 1456T>G showed 5 heterozygous variations (55.6%) in SigNH group and 4 cases of heterozygous (44.4%) and 1 case of homozygous (100.0%) variations in non-SigNH [OR: 2.50 (95%CI: 0.07-95.28; p = 0.41)]. These three variants of *UGT1A1* gene, such as 686C>A, 1091C>T, and 1456T>G, showed no significant differences in their frequencies of genotypic and allele distribution (p>0.05).



Lane 1: Ladder 100 bp
Lane 2 and 4: TT homozygote
Lane 3: TG heterozygote

Figure 4.7: PCR and restriction pattern of (3279T>G) *UGT1A1* gene. Lane 1 is a 100 bp DNA marker. Lane 2 to 4 are restriction fragment pattern of PCR products after digesting with *Dra I* and running in 3% agarose gel

In Figure 4.7, the primary PCR product for c.3279T>G was 141 bp long and included two *Dra I* sites which resulted in three restriction fragments of 141 bp, 121 bp, and 20 bp. Homozygous GG genotype was represented by one fragment (141 bp) meanwhile homozygous TT genotype was represented by two fragments (121 bp and 20 bp). PCR products of heterozygote TG was digested at three restrictions sites and produced three fragments (141 bp, 121 bp and 20 bp). However, fragment of 20 bp could not be observed clearly due to small fragment size.

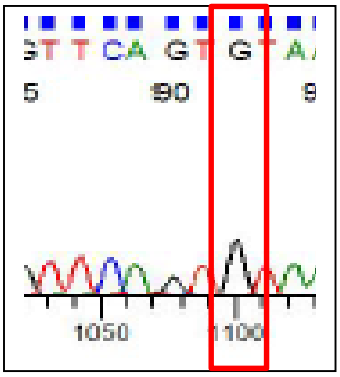
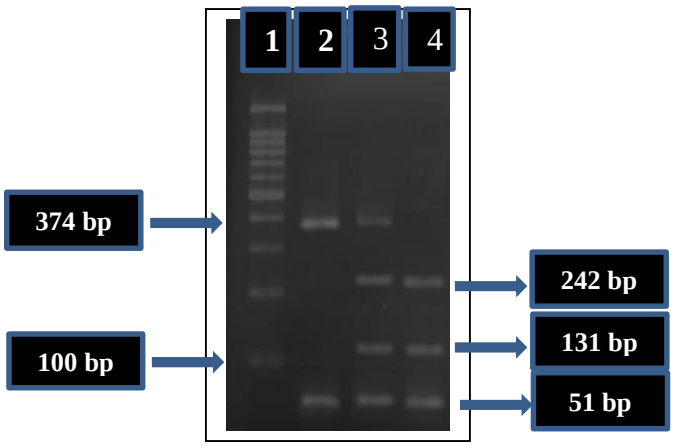


Figure 4.8: Sequence chromatogram of *UGT1A1* gene variant c.-3279T>G showing GG homozygous mutation

Figure 4.8 shows sequence chromatogram of *UGT1A1* gene homozygous variant c.-3279T>G. Variant c.-3279T>G has 152 cases of heterozygous (42.2%) and 172 cases of homozygous (70.5%) in SigNH and 208 cases of heterozygous (57.8%) and 72 cases of homozygous (29.5%) in non-SigNH group.



Lane 1: Ladder 100 bp
Lane 2: CC homozygote
Lane 3: CA heterozygote
Lane 4: AA homozygote

Figure 4.9: PCR and restriction pattern of (686C>A) *UGT1A1* gene. Lane 1 is a 100 bp DNA marker. Lane 2 to 4 are restriction fragment pattern of PCR products after digesting with *Bsr I* and running in 3% agarose gel

In Figure 4.9, the primary PCR product for 686C>A was 425 bp long and included three *Bsr I* sites which resulted in four restriction fragments of 374 bp, 242 bp, 132 bp, and 51 bp. Homozygous CC genotype was represented by two fragments (374 bp and 51 bp) meanwhile homozygous AA genotype was represented by three fragments (242 bp, 132 bp and 51 bp). PCR products of heterozygote CA was digested at three restrictions sites and produced four fragments (374 bp, 242 bp, 132 bp, and 51 bp).

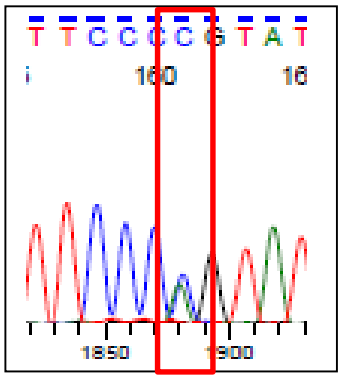
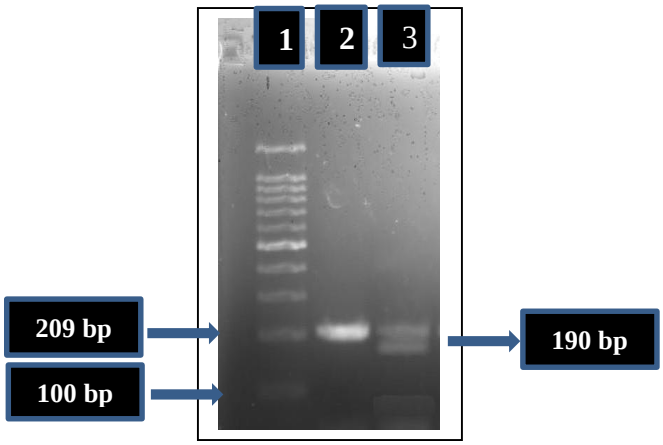


Figure 4.10: Sequence chromatogram of *UGT1A1* gene variant 686C>A showing CA heterozygous mutation

Figure 4.10 shows Sequence chromatogram of *UGT1A1* gene heterozygous variant 686C>A. The variant 686C>A showed 5 heterozygous variations (62.5%) and 1 homozygous variation (100.0%) in SigNH group and only 3 cases of heterozygous state (37.5%) detected in non-SigNH [OR: 1.20 (95%CI: 0.03-47.78; p = 0.74)].



Lane 1: Ladder 100 bp
Lane 2: CC homozygote
Lane 3: CT heterozygote

Figure 4.11: PCR and restriction pattern of (1091C>T) *UGT1A1* gene. Lane 1 is a 100 bp DNA marker. Lane 2 and 3 are restriction fragment pattern of PCR products after digesting with *Bcl I* and running in 3% agarose gel

In Figure 4.11, the primary PCR product for 1091C>T was 209 bp long and included two *Bcl I* sites which resulted in three restriction fragments of 209 bp, 190 bp, and 19 bp. Homozygous CC genotype was represented by one fragment (209 bp). PCR products of heterozygote CT was digested at two restrictions sites and produced three fragments (209 bp, 190 bp and 19 bp). However, fragment of 19 bp could not be observed clearly due to small fragment size. The 1091C>T variant showed only 3 heterozygous variations (100.0%) in SigNH group [OR: 6.00 (95%CI: 0.04-832.40; p = 0.29)].

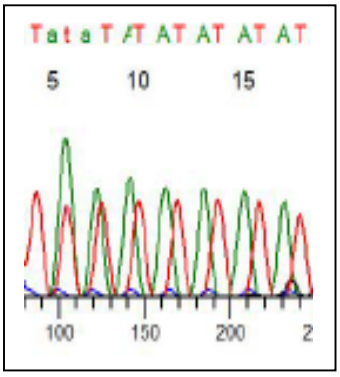


Figure 4.12: Sequence chromatogram of *UGT1A1* gene promoter A(TA)$_7$TAA homozygous mutation

Figure 4.12 shows sequence chromatogram of *UGT1A1* gene promoter homozygous A(TA)$_7$TAA homozygous mutation.

Genotypic frequency distribution of variants of *UGT1A1* gene were 211G>A (3.2%, 22/696 in SigNH and 0.5%, 2/425 in non-SigNH), 686C>A (0.9%, 6/696 in SigNH and 0.7%, 3/425 in non-SigNH), 1091C>T (0.4%, 3/696 in SigNH and 0.0% in non-SigNH), 1456T>G (0.7%, 5/696 in SigNH and 1.2%, 5/425 in non-SigNH), c.-3279T>G (46.6%, 324/696 in SigNH and 65.9%, 280/425 in non-SigNH) and promoter A(TA)$_n$TAA (14.4%, 100/696 in SigNH and 9.9%, 42/425 in non-SigNH) in our study population.

Table 4.2 compared the genotypic and allelic distribution of variants *UGT1A1* gene 211G>A, 686C>A, 1091C>T, 1456T>G and c.-3279T>G in SigNH and non-SigNH groups. Of these, variants 211G>A and c.-3279T>G showed

significant differences in their frequencies in SigNH and non-SigNH groups with OR: 6.26 (95%CI: 1.46-26.92, p = 0.02) and OR: 0.31 (95%CI: 0.22-0.43; p = 0.001) respectively. Their allelic frequencies also showed significant differences in SigNH and non-SigNH groups with OR: 5.26 (95%CI: 2.08-13.34, p = 0.04) and OR: 0.78 (95%CI: 0.66-0.93; p = 0.001). The mutant allele of 211G>A variant has higher frequency in SigNH group while mutant allele of c.-3279T>G was found to be higher frequency in non-SigNH group.

Table 4.2: Genotypic and allelic frequencies of *UGT1A1* mutations

Category	*n = 1121*		OR (95% CI)	p-value
	SigNH (TSB≥291 umol/L) (*n* = 696)	**Non-SigNH (TSB<291 umol/L) (*n* = 425)**		
211G>A				
G/G	674 (61.5%)	422 (38.5%)	1.25 (0.11-13.83)	
G/A	2 (66.7%)	1 (33.3%)	5.01 (0.30-82.74)	0.02*
A/A	20 (90.9%)	2 (9.1%)	6.26 (1.46-26.92)	
Alleles				
A	0.030	0.006	5.26 (2.08-13.34)	0.04*
686C>A				
C/C	690 (62.1%)	422 (37.9%)	1.02 (0.24-4.29)	
C/A	5 (62.5%)	3 (37.5%)	1.20 (0.03-47.78)	0.74
A/A	1 (100.0%)	0 (0.0%)	1.22 (0.04-36.54)	
Alleles				
A	0.005	0.004	1.07 (0.31-3.67)	0.90
1091C>T				
C/C	693 (62.0%)	425 (38.0%)	3.68 (0.18-73.64)	
C/T	3 (100.0%)	0 (0.0%)	6.00 (0.04-832.40)	0.29
T/T	0 (0.0%)	0 (0.0%)	1.63 (0.03-82.34)	
Alleles				
T	0.002	0	1.63 (0.25-10.68)	0.10
1456T>G				
T/T	691 (62.2%)	420 (37.8%)	1.32 (0.35-4.93)	
T/G	5 (55.6%)	4 (44.4%)	2.50 (0.07-95.28)	0.41
G/G	0 (0.0%)	1 (100.0%)	0.40 (0.01-15.24)	
Alleles				
G	0.004	0.007	0.61 (0.20-1.89)	0.41
c.-3279T>G				
T/T	372 (72.0%)	145 (28.0%)	3.51 (2.64-4.66)	
T/G	152 (42.2%)	208 (57.8%)	0.31 (0.22-0.43)	0.001*
G/G	172 (70.5%)	72 (29.5%)	1.07 (0.77-1.50)	
Alleles				
G	0.356	0.414	0.78 (0.66-0.93)	0.001*

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

Table 4.3 showed genotypic, allelic and ethnic distribution of *UGT1A1* promoter $A(TA)_nTAA$. The allele frequency between $(TA)_6/(TA)_7$ and $(TA)_7/(TA)_7$

were found to be statistically different in their frequencies between SigNH and non-SigNH groups with OR: 3.34 (95%CI: 0.09-10.26, p=0.001). All cases seen were (TA)7 and neither (TA)5 nor (TA)8 repeat was found either in SigNH or in non-SigNH. The Malay ethnic group has higher mutations OR: 1.23 (95%CI: 0.24-6.39) than the Chinese OR: 2.25 (95%CI: 0.25-20.13) and Indian ethnic groups OR: 1.82 (95%CI: 0.39-8.55) subjects.

Table 4.3: Genotypic, allelic and ethnic distribution of *UGT1A1* promoter A(TA)$_n$TAA

Category	*n = 1121*		OR (95% CI)	p-value
	SigNH (TSB≥291 umol/L) (*n*=696)	**Non-SigNH (TSB<291 umol/L) (*n*=425)**		
Promoter A(TA)$_n$ TAA				
Malay (*n* = 127)	90 (70.9%)	37 (29.1%)	1.23 (0.24-6.39)	
Chinese (*n*= 8)	6 (75.0%)	2 (25.0%)	2.25 (0.25-20.13)	
Indian (*n*= 7)	4 (11.1%)	3 (8.3%)	1.82 (0.39-8.55)	
(TA)6/(TA)6	466 (66.6%)	234 (33.4%)	1.02 (0.67-1.56)	
(TA)6/(TA)7	74 (66.1%)	38 (33.9%)	3.34 (0.09-10.26)	0.001*
(TA)7/(TA)7	26 (86.7%)	4 (13.3%)	3.26 (1.13-9.46)	
Alleles				
(TA)7	0.11	0.08	1.37 (0.97-1.96)	0.01*

* p<0.05

Note: All zero count was continuity corrected with 0.5.

Genotypic and gender distribution of variants *UGT1A1* gene 211G>A, 686C>A, 1091C>T, 1456T>G, c.-3279T>G and promoter A(TA)$_n$TAA in SigNH and non-SigNH groups are summarized in Table 4.4. There was also a slightly higher percentage of *UGT1A1* mutations in males (37.0%, 415 of 1121) compared to female (33.8%, 378 of 1121) in this study. There were no significant differences in the frequencies in all genetic variants and gender distribution (p>0.05). However, majority of mutations, such as 211G>A, 686C>A, 1456T>G and c.-3279T>G mutations were shown to be at a higher frequency in male neonates, while 1091C>T mutation was observed at a higher frequency in female patients.

Table 4.4: Genotypic and gender distribution of *UGT1A1* mutations

Category	*n = 1121*		OR (95% CI)	p-value
	Male (*n* = 574)	Female (*n* = 547)		
211G>A				
Wild Type	561 (51.2%)	535 (48.8%)	1.03 (0.47-2.28)	0.47
Mutant	13 (52.0%)	12 (48.0%)		
686C>A				
Wild Type	567 (51.0%)	545 (49.0%)	3.36 (0.70-16.27)	0.86
Mutant	7 (77.8%)	2 (22.2%)		
1091C>T				
Wild Type	573 (51.3%)	545 (48.7%)	2.10 (0.19-23.26)	0.54
Mutant	1 (33.3%)	2 (66.7%)		
1456T>G				
Wild Type	565 (50.9%)	546 (49.1%)	8.70 (1.10-68.88)	0.47
Mutant	9 (90.0%)	1 (10.0%)		
c.-3279T>G				
Wild Type	260 (50.3%)	257 (49.7%)	1.07 (0.85-1.35)	0.74
Mutant	314 (52.0%)	290 (48.0%)		
A(TA)$_n$TAA				
Wild Type	503 (46.6%)	576 (53.4%)	1.15 (0.81-1.63)	0.68
Mutant	71 (50.0%)	71 (50.0%)		

* p<0.05

Note: All zero count was continuity corrected with 0.5.

Genotypic and ethnic distribution of variants 211G>A and c.-3279T>G mutations are shown in Table 4.5 and 4.6. Malay ethnic group has higher percentage of mutations in 211G>A variant showed OR: 2.86 (95%CI: 0.21-37.99) followed by Chinese OR: 1.71 (95%CI: 0.06-50.42) and Indian OR: 1.50 (95%CI: 0.02-111.55) ethnic groups. Similar pattern has shown in c.-3279T>G in which Malay has higher number of mutations OR: 1.58 (95%CI: 1.01-2.47) than Chinese OR: 1.97 (95%CI: 0.70-5.55) and Indian OR: 0.51 (95%CI: 0.18-1.43) ethnicities, respectively. However, none of these two variants showed statistically significant difference in their frequencies between genotypic and ethnic distribution.

Table 4.5: Genotypic and ethnic distribution of *UGT1A1* 211G>A mutations

Category	*n = 25*		OR (95% CI)
	SigNH (TSB≥291 umol/L)	**Non-SigNH (TSB<291 umol/L)**	
211G>A			
Malay (*n* = 11)	10 (90.9%)	1 (9.1%)	2.86 (0.21-37.99)
Chinese (*n* = 9)	7 (77.8%)	2 (22.2%)	1.71 (0.06-50.42)
Indian (*n* = 3)	3 (100.0%)	0 (0.0%)	1.50 (0.02-111.55)
Others (n=2)	2 (100.0%)	0 (0.0%)	2.50 (0.06-103.04)

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

Table 4.6: Genotypic and ethnic distribution of *UGT1A1* c.-3279T>G mutations

Category	*n = 604*		OR (95% CI)
	SigNH (TSB≥291 umol/L)	Non-SNH (TSB<291 umol/L)	
c.-3279T>G			
Malay (*n* = 481)	253 (52.6%)	228 (47.4%)	1.58 (1.01-2.47)
Chinese (*n* = 99)	63 (63.6%)	36 (36.4%)	1.97 (0.70-5.55)
Indian (*n* = 17)	8 (47.1%)	9 (52.9%)	0.51 (0.18-1.43)
Others (n=7)	0 (0.0%)	7 (100.0%)	0.06 (0.01-1.14)

* p<0.05

Note: All zero count was continuity corrected with 0.5.

Genotypic and allelic distribution of *UGT1A1* polymorphic 214G>C mutation in the study are shown in Table 4.7. All samples of SigNH (n = 200) and non-SigNH (n = 100) showed homozygous 214G>C mutations.

Table 4.7: Genotypic and allelic distribution of *UGT1A1* polymorphic 214G>C mutation

Category	*n = 300*	
	SigNH (TSB≥291 umol/L) (*n*= 200)	Non-SigNH (TSB<291 umol/L) (*n*= 100)
214G>C		
G/G	0	0
G/C	0	0
C/C	200 (66.7%)	100 (33.3%)
Alleles		
C	1.0	1.0

* p<0.05

Note: All zero count was continuity corrected with 0.5.

4.2 Discussion

The causes of neonatal hyperbilirubinemia are multifactorial and comprise increased bilirubin production on one hand, and diminished bilirubin conjugation on the other. In recent years, many of these aetiologies have been found to have a genetic origin in various parts of the world (Agrawal, 2009). This study indicated that SigNH was 62.1% (696 of 1121) and non-SigNH was 37.9% (425 of 1121) in Malaysian neonates. Malay had the highest percentage of hyperbilirubinemia (OR: 1.27, 95% CI = 0.91-1.77, p = 0.34) while Indian had lowest number in our study population (OR: 1.42, 95% CI = 0.73-2.78, p = 0.81). The frequency of Malaysian Chinese neonates was found higher in SigNH group (OR: 1.80, 95% CI = 0.88-3.71, p = 0.34).

Wong *et al*., 2013 studied in all Malaysian main ethnic groups of neonates but their study design was case-control study. In their study, Malay ethnic group showed significant higher percentage in case group compared with other ethnic groups (p = 0.004) but there is no significant difference in gender distribution between case and control groups (p = 0.08).

In Malaysia, Teh *et al*., 2012 studied the demographic characteristics on all Malaysian main ethnic groups but they studied on adult populations. Their study involved adult healthy unrelated volunteers and the ethnic groups distribution was not significantly difference (Malay = 100, Chinese = 104 and Indian = 102).

In one study done in China (Chen *et al*., 2008) showed that there was no statistical difference in gender distribution in their study (P>0.05). Another study done in Taiwan (Zhou *et al*., 2014) reported similar result stated that there was no significant difference in gender (52.4% male vs 47.6% female) in their hyperbilirubinemic neonates. Moreover, a study done in India (Tiwari *et al*., 2014) observed that there was also no significant difference in gender distribution (63.7% male vs 36.3% female) in jaundiced neonates.

The recent study result showed that the majority of samples were Malay, second highest number was from Chinese and the least number was from Indian. This distribution is similar to that of the Malaysian ethnic population although the present study design was not a population study and it was not stratified based on ethnicity. In addition, the sampling method used in the study was 'Universal Sampling' as we recruited all hospitalized jaundiced neonates who provided consent and are eligible following the inclusion criteria.

The recent study design is different from previous Malaysia studies of *UGT1A1* gene mutations. All our patients were hyperbilirubinemic neonates and this research mainly studied on SigNH neonates while majority of previous Malaysia studies were done in hyperbilirubinemic case and non-hyperbilirubinemic control neonates. The results of the present study showed that 70.7% (793/1121) of the main ethnic groups of neonates carried at least one *UGT1A1* gene mutation. In

our study, the frequency of mutations of *UGT1A1* gene variants was higher in SigNH group (58.0%, 460/793) compared to non-SigNH group (42.0%, 333/793).

In addition, the frequency of heterozygous state was higher (62.4%, 495/793) than homozygous state (37.6%, 298/793). The most common mutation was nt c.-3279T>G (76.2%) followed by TA promoter repeat region (17.9%) and nt 211G>A (3.2%) in our study group. Furthermore, *UGT1A1* variants 686 C>A, 1091C>T and 1456T>G were studied and no one has reported these variants before in Malaysia within our best knowledge.

No significant variants

Variant 686 C>A of *UGT1A1* gene mutation was commonly reported in Asians (Ando *et al*., 2000, Bosma *et al*., 1995). This study found nt 686 C>A mutation of 0.9% (6 of 696) in SigNH and 0.7% (3 of 425) in non-SigNH babies and this mutation was detected in Chinese and Malay ethnic groups which were 1.1% and 0.6% respectively, but none of the Indian neonates had this genotype mutation. These findings are consistent with Teh *et al* study (2012) in which 686 C>A was found in Malay population where the frequencies were less than 3% in both Chinese and Malay population and they did not detect in Malaysian Indian subjects. Similar finding was found in Tiwari *et al* study (2014) where 686C>A mutation was not detected in Indian newborn babies. The study by Wu *et al*., 2015 reported that heterozygous 686C>A mutation was identified in Han ethnic group (1 in 218) and therefore statistical analysis was not possible.

In the present study, variant 1091C>T mutation of *UGT1A1* gene was found only in 0.4% (3 of 696) of SigNH group and this variant was detected in Malay and Chinese ethnic groups but not detected in Malaysian Indian newborn babies. Tiwari *et al* study (2014) found similarly where variant 1091C>T mutation was not detected in Indian newborn babies. The study by Huang *et al* (2008) stated that this 1091C>T variant was detected both in cases and controls groups but not a significant risk factor to develop severe neonatal jaundice in Taiwanese newborns (p=0.58). However, our study detected this mutation with higher frequency in significant neonatal jaundice in Malaysian newborns.

Variant 1456T>G mutation of *UGT1A1* gene was found in 0.7% (5 of 696) of SigNH group and 0.9% (4 of 425) in non-SigNH group and this variant was detected in Malays and Chinese but not detected in Indian newborn babies. This finding was consistent with Tiwari *et al* study (Tiwari et al., 2014) where variant 1456T>G mutation was not detected in Indian newborns study. The study by Maruo *et al* (2000) stated that heterozygous 1456T>G mutation was found in Japanese breastfed infants (1 of 17) and this mutation may be an associated condition of breast milk jaundice (BMJ) and that constituent(s) of breast milk may generate BMJ in newborns who have those defects.

Although there were no statistical differences in their frequencies of *UGT1A1* gene variant 686 C>A, variant 1091C>T and variant 1456T>G (p=>0.05)

in SigNH and non-SigNH, this study can show the mutation patterns of these variants in significant hyperbilirubinemic Malaysian neonates. Moreover, these three variants mutation results can be reported as new reports of significant neonatal hyperbilirubinemia in Malaysia although we detected few mutation cases.

Significant variants

This study observed that variant c.-3279T>G mutation of *UGT1A1* gene was 47.6% (331 of 696) in SigNH and 64.2% (273 of 419) in non-SigNH and this variant was detected in all main Malaysian ethnic groups. In the study, Malay and Chinese have higher frequencies of this variant mutation which were 58.4% and 52.1% respectively compared to 47.2% in Indian ethnic group. Yusoff *et al* study (2010) found that c.-3279 T>G was related with neonatal hyperbilirubinemia and the genotype distribution between both control and patient groups were significantly different (p=0.003): the incidence of homozygosity for c.3279T>G was much greater in cases than those in controls and the allele frequency is 33.0% in Malay newborns.

The study by Innocenti *et al* (2005) showed that the allele frequency of c.3279 T>G is 44.0% in Whites and 34.0% in Asians. The study by Huang *et al* (2008) observed c.-3279 T>G was only found in hyperbilirubinemic Taiwanese subjects accounted for 87.1% (155 of 178), and none was identified in the control group. It is possible that *UGT1A1* maturation process activities are related with the pathway activating the promoter and mixture of cis- or trans-acting factors analysis

of the *UGT1A1* promoter may offer an evidence to understanding the maturation pathogenesis of this gene activity (Yusoff *et al*., 2006). In Sugatani *et al* study (2002), c.-3279T>G mutations reduced the transcriptional action to 62% of normal of the *UGT1A1* gene. However, the exact pathogenesis of c.-3279T>G variant mutation in hyperbilirubinemia is debatable (Tiwari, 2014).

The study by Huang indicated that the development of hyperbilirubinemia has been associated with the mutation of *UGT1A1* c.-3279T>G. This result revealed that the c.-3279T>G variant is worth studying for each ethic population (Huang *et al*., 2008). Coexpression of *UGT1A1* gene coding sequence and promoter region as well as *G6PD* mutations with *SLCO1B1* polymorphisms can suggestively increase hyperbilirubinemia hazard (Kaplan *et al*., 1997, Watchko *et al*., 2009).

Neonatal hyperbilirubinemia in western people was found to be associated with *UGT1A1* gene homozygygous $A(TA)_7TAA$ variation in promoter region (Lin *et al*., 2008). From the sequencing analysis of *UGT1A1* gene promoter area [A $(TA)_n$ TAA] mutation, the prevalence of mutation in the TA promoter region is 14.4% (100 of 696) in SigNH and 9.9% (42 of 425) in non-SigNH and this mutation was detected in all three Malaysian ethnic groups. All cases in this study were $A(TA)_7TAA$.

The *UGT1A1* gene promoter area allele was first described by Beutler *et al* in 6.9% of healthy subjects of North and Central America with varying degrees of

Aferican ancestry. This study showed that there is an inverse relationship between number of TA repeats and the activity of promoter through the range of 5 to 8 TA repeats. (TA)8 allele is very rare in Caucasians (Beutler *et al*., 1998). The relationship between neonatal jaundice may vary between racial groups and the site of *UGT1A1*variant (Chou *et al*., 2011). Indians and Malays have higher frequencies of A$(TA)_7$TAA repeat which were 19.4% and 19.3% respectively compared to 5.4% in Chinese newborns. Our finding is similar to that of Yusoff *et al* study (2010) where this study was detected that the prevalence of *UGT1A1**28 (TA promoter) in a Malays group was 1.5%. Azlin *et al* study (2011) found that low occurrence in the Malays and this has been revealed to be frequent mutation affecting Gilbert's syndrome in Caucasians.

Teh *et al* study done in three major ethnic groups of Malaysia reported that Indian and Malays have twofold greater occurrence of homozygote 7TA/7TA (*UGT1A1**28), respectively compared to the Chinese. Nevertheless, the distribution of 211G>A (*UGT1A1**6) and 686C>A (UGT1A1*27) revealed no significant differences between them (Teh *et al*., 2012). African American and Caucasian population did not detect *UGT1A1**27, but this mutation was found in the Malaysian Chinese (2.0%) and Malaysian Malays (3.33%). This study concluded that the Malaysian population has inconsistency in ethnic groups in the incidence of (TA) promoter mutation (Teh *et al*., 2012).

Gupta *et al* study (2015) reported that more than 90% of their study patients with unconjugated hyperbilirubinemia of Gilbert syndrome variety had the homozygous $(TA)_7$ promotor genotype. But Teh *et al* study (2012) found *UGT1A1**28 variants were observed in all main Malaysian races and about 24.5 to 39.2% of adults Malaysian were heterozygous of this variant. In their research, Malay and Indian have twofold greater incidence of homozygous of *UGT1A1**28 which is 8.0 and 8.8% respectively compared to only 4.9% in the Malaysian Chinese. The data from the present study showed that 74 heterozygous and 26 homozygous cases in SigNH and 4 homozygous and 38 heterozygous cases in non-SigNH groups respectively were detected.

Variant 211 G>A (Gly71Arg) mutation in the axon 1 of *UGT1A1* gene has been often reported in hyperbilirubinemic individuals, the present study found this mutation in 3.2% (22 of 696) of SigNH and 0.7% (3 of 425) of non-SigNH babies and indicating that neonates who carry variant 211 in the *UGT1A1* gene are at a higher risk of developing significant jaundice. The variant 211G>A mutation was highest in Malay ethnic group (44.0%, 11/25) followed by Chinese (36.0, 9/25), Indians (12.0%, 3/25) and others (mixed ethnic group) (8.0%, 2/25). Different finding was reported by D'Silva *et al* study (2012) in which G71R mutation of the *UGT1A1* gene was not associated with neonatal jaundice in Indian newborns. Interestingly, the present study observed this 211G>A variant mutation in Indian newborns and all mutation cases were only from SigNH group. Hence, this study

result is the first report of the *UGT1A1* gene 211 G>A variant mutation in Malaysian Indian newborns with significant neonatal hyperbilirubinemia.

Variant 211G>A is associated with reduced isenzyme activity, ranging from 60% in heterozygous state to 14-32% of normal levels in homozygous state (Koiwai, 1995). Neonatal hyperbilirubinemia was associated with the high occurrence of *UGT1A1* gene G211A in Taiwanese, Koreans, and Japanese studies (Huang *et al*., 2004). The study by Chou stated that infants with *UGT1A1* genotypes nt G211A variation had greater incidence of hyperbilirubinemia and greater peak serum bilirubin levels than GG wild types. This study suggested that early-onset neonatal breastfeeding jaundice are less susceptible to develop jaundice than infants who carry the *UGT1A1*nucleotide G211A mutation (Chou *et al*., 2011). Moreover, in East Asians, missense mutations in the *UGT1A1* gene nt G211A are the most common (Agrawal *et al*., 2009).

The *UGT1A1* genotypes nt G211A variation is a major cause of breast milk jaundice in East Asia (Maruo, 2014). Chen *et al*., 2007 stated that neonates with the normal controls and promoter variation had higher peak levels within 7 days and lower bilirubin levels at 72 hours compared to those with *UGT1A1* gene variation of nucleotide 211 (c.211G>A). The neonates who carry promoter polymorphism and c.211G>A had OR:1.2, and OR:2.4 respectively. This study confirmed that *UGT1A1* gene c.211G>A was a risk condition for the progress of neonatal jaundice.

This study also suggested to conduct a larger population study for further evaluation.

One study in Malaysia (Azlin *et al*., 2011) detected the nt 211G>A variant mutation with higher frequency showing incidence of 13.0% in severe hyperbilirubinemia newborns and 4.0% in control group. Similarly, the study by Boo *et al* (2009) found that the prevalence of nt G211A in the hyperbilirubinemic newborns was 39.2% and even the non-hyperbilirubinemic neonate group carried this mutation 25.6%, p = 0.04 in their study. The study done in China (Wu, 2015) reported that nt 211G>A mutations was found 33.9% (74/218) in their case group. The data from India study (Agrawal, 2009) showed that this 211G>A variant mutation was not detected in either in hyperbilirubinemics or controls.

Although statistically not significant difference in frequency of *UGT1A1* variant 211G>A mutation and small percentage of prevalence in neonatal jaundice cases in Malaysian Indian newborns compared with other Malaysian ethnic groups, the present study found out this mutation is associated with SigNH group. Moreover, interestingly, the homogenous mutation of nt 211 G>A mutation with Indian neonate presented with severe degree of neonatal hyperbilirubinemia (TSB >342 mmol/L).

Furthermore, the sequencing analysis results of the randomly selected 300 samples of SigNH (n = 200) and non-SigNH (n = 100) showed homozygous variant

214G>C (Alu72Pro) mutation in which CCA at the codon position 72 of the axon 1 of *UGT1A1* gene in place of the codon GCA. Our finding is consistent with Agrawal *et al* study conducted in India in which this mutation was detected in all control and case subjects. It has been seen that mutations found in exon 1 might reduce the affinity of the ligand, bilirubin, for the catalytic site in the amino-terminal half of the molecule, which is encoded by exon 1 of the UGT1A1 gene (Agrawal *et al*., 2009). This single nucleotide polymorphism has not been reported earlier in Malaysia. This results also would be the first report in Malaysia of this polymorphic variant in Significant neonatal hyperbilirubinemia cases.

4.3 Conclusion

Malay neonates had the highest percentage in our study population followed by Chinese and Indian ethnic groups. Interestingly, although Malay has the highest frequency in overall, Chinese newborns showed the highest frequency in SigNH group. There was no significant difference in the gender and ethnic distribution between SigNH and non-SigNH in our study population.

In conclusion, *UGT1A1* gene variants 3279T>G, $A(TA)_nTAA$ promoter area and nucleotide G211A mutations were found to be most common mutations in the main ethnic groups and all the identified six variants showed higher frequencies of mutations in SigNH group in this study. Furthermore, the odds ratios (ORs), adjusted for covariates and logistic regression for the variants 3279T>G, $A(TA)_nTAA$ promoter area and allele G211A mutations of *UGT1A1* gene were

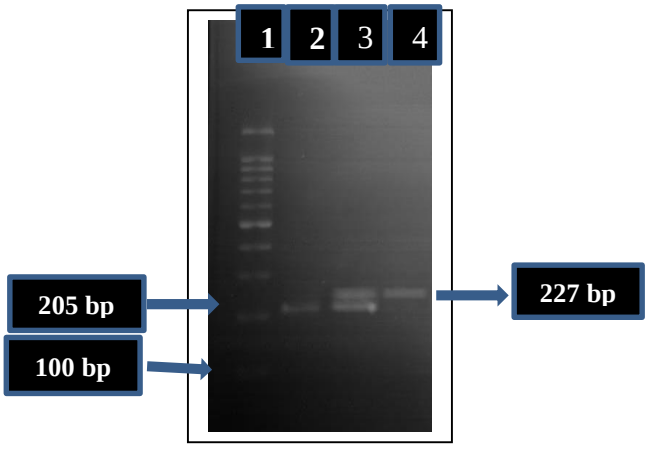
statistically significant in genotypic distribution between SigNH and non-SigNH (p = <0.001, p = <0.001 and p = <0,02) and are significant predictor for SigNH and non-SigNH groups. Moreover, this study can report the polymorphic variation of *UGT1A1* gene nt 214G>C in Significant neonatal hyperbilirubinemia. The present study demonstrates that genetic risk factors modulate hyperbilirubinemia risk in newborns. Determination of *UGT1A1* genes variants 3279T>G, $A(TA)_nTAA$ promoter area and nucleotide G211A is recommended in investigation of unconjugated hyperbilirubinemia in neonates.

CHAPTER 5

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (*G6PD*) GENE MUTATIONS

5.1 Results

Out of 1121 subjects studied, 3.1% (35/1121) were found to have *G6PD* mutation. From the eight variants of *G6PD* gene identified, the present study detected 5 variant mutations such as nt 1388G>A (60.0%), nt 871G>A (17.1%), nt 487G>A (14.3%), nt 1376G>T (5.7%) and nt 1003G>A (2.9%).



Lane 1: Ladder 100 bp
Lane 2: AA genotype
Lane 3: GA genotype
Lane 4: GG genotype

Figure 5.1: PCR and restriction pattern of (nt 1388G>A) *G6PD* gene. Lane 1 is a 100 bp DNA marker. Lane 2 to 4 are restriction fragment pattern of PCR products after digesting with *Nde I* and running in 3% agarose gel

In Figure 5.1, the primary PCR product for 1388G>A was 227 bp long and included two *Nde I* sites which resulted in three restriction fragments of 227 bp, 205 bp, and 22 bp. Genotype GG was represented by one fragment (227 bp) while AA was represented by two fragments (205 bp and 22 bp). PCR products of GA was digested at two restrictions sites and produced three fragments (227 bp, 205 bp, and 22 bp). However, fragment of 22 bp could not be observed clearly due to small fragment size.

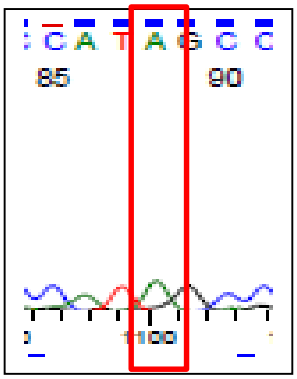
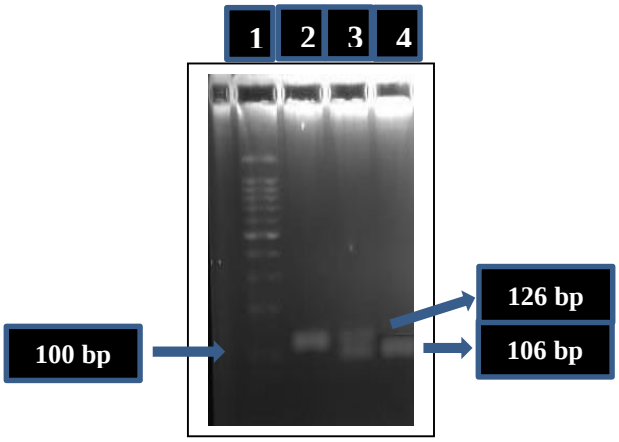


Figure 5.2: Sequence chromatogram of *G6PD* gene variant 1388G>A showing hemizygous mutation

Figure 5.2 shows sequence chromatogram of *G6PD* gene hemizygous of variant 1388G>A. The status of *G6PD* gene at nucleotide 1388G>A in 696 DNA samples of significant neonatal hyperbilirubinemic (SigNH) newborns were 17 cases [13 for the male hemizygous variation (13/17, 76.5%) and 4 for the female heterozygous variation (4/17, 23.5%)]. In 425 DNA samples of non-significant hyperbilirubinemic (non-SigNH) newborns were 4 cases for female heterozygous

variation only (OR: 2.02, 95% CI = 0.65-6.22, p = 0.14). Nucleotide 1388G>A mutation was found in both male (13/21) and female (8/21, all heterozygous) neonates.



Lane 1: Ladder 100 bp
Lane 2: GG genotype
Lane 3: GA genotype
Lane 4: AA genotype

Figure 5.3: PCR and restriction pattern of (nt 871G>A) *G6PD* gene. Lane 1 is a 100 bp DNA marker. Lane 2 to 4 are restriction fragment pattern of PCR products after digesting with *Xba I* and running in 3% agarose gel

In Figure 5.3, the primary PCR product for 871G>A was 126 bp long and included two *Xba I* sites which resulted in three restriction fragments of 126 bp, 106 bp, and 20 bp. Genotype GG was represented by one fragment (126 bp) while AA was represented by two fragments (106 bp and 20 bp). PCR products of GA was digested at two restrictions sites and produced three fragments (126 bp, 106 bp and

20 bp). However, fragment of 20 bp could not be observed clearly due to small fragment size.

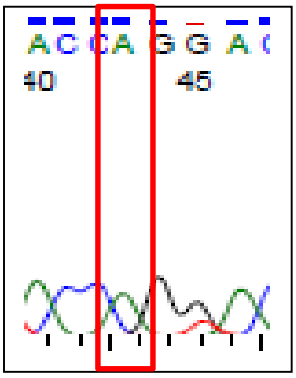
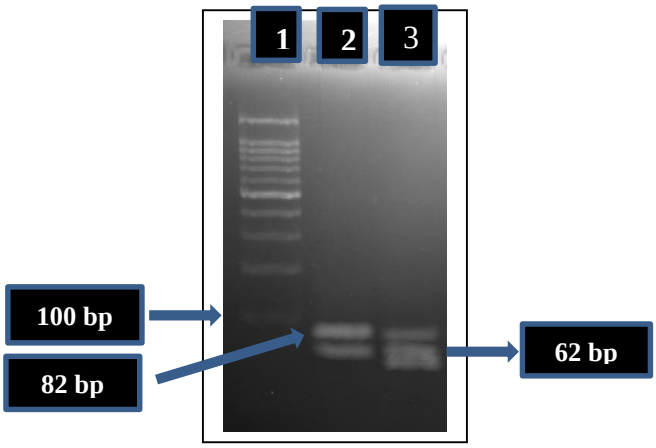


Figure 5.4: Sequence chromatogram of *G6PD* gene variant 871G>A showing hemizygous mutation

Figure 5.4 shows sequence chromatogram of *G6PD* gene hemizygous of variant 871G>A. A total of 3 male hemizygous and 1 female heterozygous in SigNH and 2 female heterozygous variations in nucleotide 871G>A were detected in non-SigNH respectively (OR: 1.22, 95% CI = 0.22-6.70, p = 1.00). Variant 871G>A mutation was detected in both male (3/6) and female (3/6, all heterozygous) neonates.



Lane 1: Ladder 100 bp
Lane 2: GG genotype
Lane 3: GA genotype

Figure 5.5: PCR and restriction pattern of (nt 487G>A) *G6PD* gene. Lane 1 is a 100 bp DNA marker. Lane 2 and 3 are restriction fragment pattern of PCR products after digesting with *Alu I* and running in 3% agarose gel

In Figure 5.5, the primary PCR product for 487G>A was 214 bp long and included three *Alu I* sites which resulted in four restriction fragments of 82 bp, 62 bp, 60 bp, and 20 bp. Genotype GG was represented by two fragments (82 bp and 60 bp). PCR products of GA was digested at three restrictions sites and produced four fragments (82 bp, 62 bp, 60 bp, and 20 bp). However, fragment of 20 bp could not be observed clearly due to small fragment size.

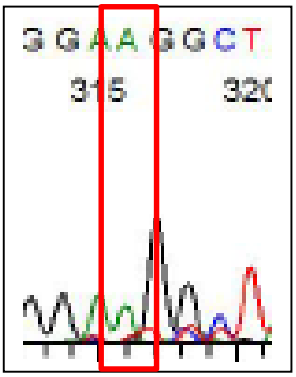
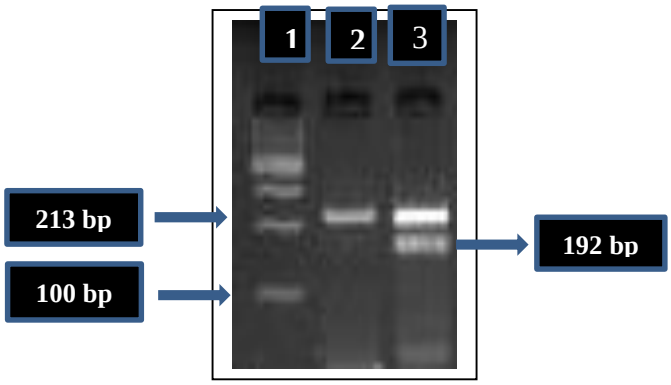


Figure 5.6: Sequence chromatogram of *G6PD* gene variant 487G>A showing hemizygous mutation

Figure 5.6 shows sequence chromatogram of *G6PD* gene hemizygous of variant 487G>A. The five male hemizygous variation in nucleotide 487G>A were found only in SigNH group (OR: 1.55, 95% CI = 0.02-111.55, p = 0.22). This variant 487G>A mutation was found only in male neonates.



Lane 1: Ladder 100 bp
Lane 2: GG genotype
Lane 3: GT genotype

Figure 5.7: PCR and restriction pattern of (nt 1376G>T) *G6PD* gene. Lane 1 is a 100 bp DNA marker. Lane 2 and 3 are restriction fragment pattern of PCR products after digesting with *Xho I* and running in 3% agarose gel

In Figure 5.7, the primary PCR product for 1376G>T was 213 bp long and included two *Xho I* sites which resulted in three restriction fragments of 213 bp, 192 bp, and 21 bp. Genotype GG was represented by one fragment (213 bp). PCR products of GT was digested at two restrictions sites and produced three fragments (213 bp, 192 bp, and 21 bp). However, fragment of 21 bp could not be observed clearly due to small fragment size.

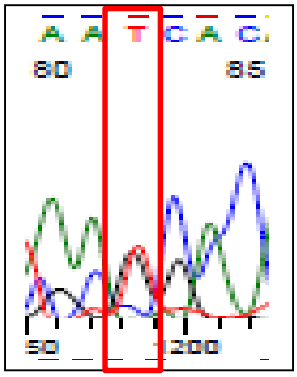
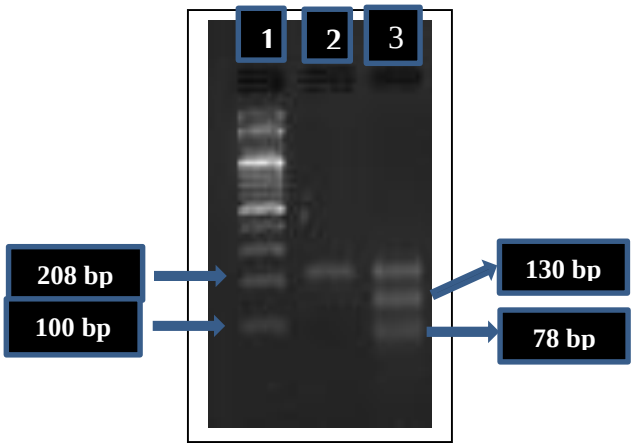


Figure 5.8: Sequence chromatogram of *G6PD* gene variant 1376G>T showing hemizygous mutation

Figure 5.8 shows sequence chromatogram of *G6PD* gene hemizygous of variant 1376G>T. Only two male hemizygous TT variations in nucleotide 1376G>T (OR: 4.00, 95% CI = 0.03-591.90, p = 0.53) were detected in the SigNH group of male neonates.



Lane 1: Ladder 100 bp
Lane 2: GG genotype
Lane 3: GA genotype

Figure 5.9: PCR and restriction pattern of (nt 1003G>A) *G6PD* gene. Lane 1 is a 100 bp DNA marker. Lane 2 and 3 are restriction fragment pattern of PCR products after digesting with *Bst XI* and running in 3% agarose gel

In Figure 5.9, the primary PCR product for 1003G>A was 208 bp long and included two *Bst XI* sites which resulted in three restriction fragments of 208 bp, 130 bp, and 78 bp. Genotype GG was represented by one fragment (208 bp). PCR products of GT was digested at two restrictions sites and produced three fragments (208 bp, 130 bp, and 78 bp).

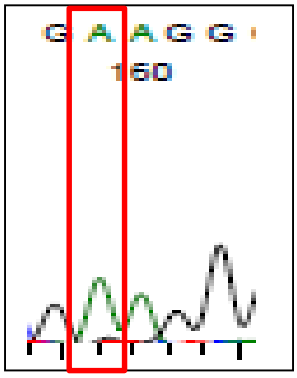


Figure 5.10: Sequence chromatogram of *G6PD* gene variant 1003G>A showing hemizygous mutation

Figure 5.10 shows sequence chromatogram of *G6PD* gene variant 1003G>A. One male hemizygous variation in nucleotide 1003G>A were found in SigNH group (OR: 2.00, 95% CI = 0.01-357.39, p = 1.00) of male neonate.

Table 5.1 showed genotypic and allelic distribution of *G6PD* mutations in the study. Genotypic frequency distribution of variants of *G6PD* gene were 1388G>A (2.4%, 17/696 in SigNH and 0.9%, 4/425 in non-SigNH), 871G>A (0.6%, 4/696 in SigNH and 0.5%, 2/425 in non-SigNH), 487G>A (0.7%, 5/696 in SigNH and 0.0% in non-SigNH), 1376G>T (0.3%, 2/696 in SigNH and 0.0% in non-SigNH) and 1003G>A (0.1%, 1/696 in SigNH and 0.0% in non-SigNH) in our study population. There is no significant difference in their frequencies between genotypic and allele distributions of all detected five mutated variants of *G6PD* gene in our study (p>0.05). We did not find any mutations of nucleotide 563 C>T, 383T>C, and 131 C>G in the study population.

Table 5.1: Genotypic and allelic frequencies of *G6PD* mutations

Category	*n = 1121*		OR (95% CI)	p-value
	SigNH (TSB≥291 umol/L) (n = 696)	**Non-SigNH (TSB<291 umol/L) (n = 425)**		
1388 G>A (Kaiping)				
G/G	679 (61.7%)	421 (38.3%)	2.02 (0.65-6.22)	
G/A	4 (50.0%)	4 (50.0%)	0.04 (0.01-0.89)	0.14
A/A	13 (100.0%)	0 (0.0%)	4.96 (0.26.94.06)	
Alleles				
A	0.02	0.005	2.59 (0.97-6.90)	0.11
871 G>A (Viangchan)				
G/G	692 (62.1%)	423 (37.9%)	1.22 (0.22-6.70)	
G/A	1 (66.7%)	2 (33.3%)	0.08 (0.01-3.87)	1.00
A/A	3 (100.0%)	0 (0.0%)	3.67 (0.18-73.47)	
Alleles				
A	0.005	0.002	1.02 (0.24-4.27)	0.76
487G>A (Mahidol)				
G/G	691 (61.9%)	425 (38.1%)	1.84 (0.19-17.75)	
G/A	0 (100.0%)	0 (0.0%)	1.50 (0.02-111.55)	0.22
A/A	5 (100.0%)	0 (0.0%)	2.46 (0.11-54.69)	
Alleles				
A	0.007	0	2.15 (0.45-10.38)	0.10
1376G>T (Canton)				
G/G	694 (62.0%)	425 (38.0%)	1.22 (0.11-13.52)	
G>T	0 (0.0%)	0 (0.0%)	0.25 (0.01-36.99)	0.53
T>T	2 (100.0%)	0 (0.0%)	4.00 (0.03-591.90)	
Alleles				
T	0.003	0	1.09 (0.18-6.53)	0.10
1003G>A (Chatham)				
G/G	695 (62.1%)	425 (37.9%)	1.64 (0.10-26.28)	
G/A	0 (0.0%)	0 (0.0%)	0.50 (0.01-89.35)	1.00
A/A	1 (100.0%)	0 (0.0%)	2.00 (0.01-357.39)	
Allele				
A	0.001	0	1.23 (0.15-10.20)	1.00

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

The predominant variation in the *G6PD* gene was the G to A substitution at nucleotide 1388 (*G6PD* Kaiping with both female heterozygous and male hemizygous states), followed by the G to A substitution at nucleotide 871 (*G6PD* Viangchan with both female heterozygous and male hemizygous states), the G to A substitution at nucleotide 487 (*G6PD* Mahidol with male hemizygous states), the only hemizygous mutation of G to T substitution at nucleotide 1376 (*G6PD* Canton), and also the only male hemizygous mutation of G to A substitution at nucleotide 1003 (*G6PD* Chatham).

The demographic distribution of *G6PD* mutations in the study is summarized in Table 5.2. This study found *G6PD* gene mutations in the Malay and Malaysian Chinese ethnic groups but not in the Malaysian Indian neonates. Higher number of mutations was seen in Malay (91.4%, 32/35) (OR: 2.70, 95% CI = 0.20-35.75) followed by Chinese (8.6%, 3/35) (OR: 2.00, 95% CI = 0.02-198.29) ethnicity. In addition, the frequency of *G6PD* mutations was found higher in male 68.6% (24/35) compared to female neonates 31.4% (11/35) (OR: 1.11, 95% CI = 0.17-7.22).

Table 5.2: Demographic characteristic distribution of *G6PD* mutations

Category	*n = 35*		OR (95% CI)
	SigNH (TSB≥291 umol/L) (*n* = 29)	**Non-SigNH (TSB<291 umol/L) (*n* = 6)**	
Gender			
Female	9 (81.8%)	2 (18.2%)	1.11 (0.17-7.22)
Male	20 (83.3%)	4 (16.7%)	
Races			
Malay	27 (84.4%)	5 (15.6%)	2.70 (0.20-35.75)
Chinese	2 (66.7%)	1 (33.3%)	2.00 (0.02-198.29)
Indian	0 (0.0%)	0 (0.0%)	
Others	0 (0.0%)	0 (0.0%)	

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

Table 5.3 shows genotypic and gender distribution of *G6PD* gene mutations. Variants mutation of *G6PD* gene 1388G>A, 487G>A, 1376G>T and 1003G>A were found to have higher frequencies in male.

Table 5.3: Genotypic and gender distribution of *G6PD* mutations

Category	*n = 1121*		OR (95% CI)	p-value
	Male (*n* = 574)	Female (*n* = 547)		
1388G>A				
Wild Type	561 (51.0%)	539 (49.0%)	1.56 (0.64-3.80)	0.11
Mutant	13 (61.9%)	8 (38.1%)		
871G>A				
Wild Type	571 (51.2%)	544 (48.8%)	0.95 (0.19-4.74	0.76
Mutant	3 (50.0%)	3 (50.0%)		
487G>A				
Wild Type	569 (51.0%)	547 (49.0%)	9.61 (0.52-176.39)	0.10
Mutant	5 (100.0%)	0 (0.0%)		
1376G>T				
Wild Type	572 (51.1%)	547 (48.9%)	3.83 (0.17-85.02)	0.10
Mutant	2 (100.0%)	0 (0.0%)		
1003G>A				
Wild Type	573 (51.1%)	547 (48.9%)	1.91 (0.06-57.03)	1.00
Mutant	1 (100.0%)	0 (0.0%)		

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

Table 5.4 shows genotypic and ethnic distribution of *G6PD* gene mutations. All *G6PD* variant mutations were detected in Malay hyperbilirubinemic neonates while Chinese neonates are only associated with mutations of variants 1388G>A, 871G>A and 1376G>T.

Table 5.4: Genotypic and ethnic distribution of *G6PD* mutations

Category	*n = 1121*		OR (95% CI)
	SigNH (TSB≥291 umol/L)	Non-SigNH (TSB<291 umol/L)	
1388G>A			
Malay	16 (80.0%)	4 (20.0%)	2.00 (0.06-7084)
Chinese	1 (100.0%)	0 (0.0%)	2.00 (0.01-357.39)
Indian	0 (0.0%)	0 (0.0%)	
Others	0 (0.0%)	0 (0.0%)	
871G>A			
Malay	4 (80.0%)	1 (20.0%)	8.00 (0.14-454.90)
Chinese	0 (0.0%)	1 (100.0%)	0.50 (0.01-89.35)
Indian	0 (0.0%)	0 (0.0%)	
Others	0 (0.0%)	0 (0.0%)	
487G>A			
Malay	5 (100.0%)	0 (0.0%)	10.00 (0.08-931.6)
Chinese	0 (0.0%)	0 (0.0%)	
Indian	0 (0.0%)	0 (0.0%)	
Others	0 (0.0%)	0 (0.0%)	
1376G>T			
Malay	1 (100.0%)	0 (0.0%)	1.00 (0.01-121.63)
Chinese	1 (100.0%)	0 (0.0%)	2.00 (0.01-357.39)
Indian	0 (0.0%)	0 (0.0%)	
Others	0 (0.0%)	0 (0.0%)	
1003G>A			
Malay	1 (100.0%)	0 (0.0%)	2.00 (0.01-357.39)
Chinese	0 (0.0%)	0 (0.0%)	
Indian	0 (0.0%)	0 (0.0%)	
Others	0 (0.0%)	0 (0.0%)	

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

5.2 Discussion

Glucose-6-phosphate dehydrogenase (G6PD) is an ezzyme with considerable role in the metabolism of red blood cells (Mondal, 2012). The recent study design is different from previous Malaysian *G6PD* newborns and adult

studies. This research studied on SigNH neonates and the molecular characterisation of *G6PD* gene was done without knowing G6PD screening test results while almost all previous Malaysian studies were done after G6PD enzyme level testing. Moreover, all the neonates in this study population were hyperbilirubinemic while other studies were mostly case-control study.

The result of the present study showed that 3.1% (35/1121) of the main ethnic groups of hyperbilirubinemic neonates carried a *G6PD* mutation. In the study, the frequency of mutations of *G6PD* gene variants was detected higher in SigNH group (82.9%, 29/35) compared to non-SigNH group (17.1%, 6/35). In addition, the frequency of male hemizygous state was higher (68.6%, 24/35) than female heterozygous state (31.4%, 11/35). In addition, all the male hemizygous cases were from SigNH group. However, female heterozygous cases were detected in the SigNH (5/11) and non-SigNH (6/11) groups.

The present study identified hemizygous mutations of *G6PD* gene in male neonates and only heterozygous mutations in female neonates. Majority of male hemizygous mutations were from SigNH group. Although *G6PD* deficient males have more severe clinical presentations than heterozygous females, some female patients progress acute severe hemolytic anaemia (Lim *et al*., 2005). Numerous heterozygotes female with incomplete enzymatic action will not be observed by biochemical screening methods and female heterozygotes possibly will have intermediate action, but this can range from normal to defective form. These

individuals can only be identified by molecular screening methods (Kaplan and Hammerman, 2010).

Although the data from this study were not statistically significant different in SigNH and non-SigNH, *G6PD* mutations were more commonly found in male (24/35, 68.6%) compared to female (11/35, 31.4%). This finding was in line with Wang *et al* study (2008) where they showed that there was no significant difference in the proportion of Malay newborns with *G6PD* mutation between the two gender groups (females 11.6%, males 15.0%) ($p = 0.5$). The study by Sulaiman (2013) reported that the prevalence of *G6PD* deficiency was 4.59% (4 of 87), all of whom were Malay males. The study by Yang (2015) stated that the overall incidence of *G6PD* deficiency in their China cohort study was 2.68% and the incidence of female population was 2.03% and in male population was 3.22%, respectively.

The part of genetic polymorphisms and mutations in the pathogenesis of jaundice is important in general and definitely in *G6PD*-deficiency related hyperbilirubinemia, is increasingly obvious (Kaplan and Hammerman, 2002). In *G6PD* deficient individuals, exposure to environmental influences may precipitate severe hemolysis, overwhelm the hepatic bilirubin conjugation processes and result in extreme hyperbilirubinemia (Kaplan and Hammerman, 2010).

In this study, Malay ethnic group has higher prevalence of mutations than Chinese neonates. This finding was consistent with the previous Malaysia studies

and these studies found that *G6PD* deficiency was detected to be more prevalent amongst Malay and Malaysian Chinese individuals and less among Malaysian Indians (Ainoon, 2002 and Ainoon, 2003). Ainnon *et al* study (2002) reported that the overall prevalence of *G6PD* deficiency among males is 5.3% with an ethnic breakdown of 7.2%, 4.6%, and 2.7% among Chinese, Malays and Indians correspondingly.

Neonatal glucose-6-phosphate dehydrogenase assessment ought to be done in those with a previous history of neonatal jaundice in family, or in those who develop jaundice in the first day of life (Chen *et al*., 2008). *G6PD* deficient infants with hyperbilirubinemia results from modestly increased hemolysis (Kaplan *et al*., 1996, Kaplan *et al*., 2001) in combination preferentially diminished bilirubin conjugation (Kaplan *et al*., 2006). In Hongkong and Singapore, 55% and 50% of cases of kernicterus were because of *G6PD* deficiency (Wong, 1965, Lai *et al*., 1968). Molecular characterisation in Thai adults showed an incidence of 14.2% and the nucleotide G871A (*G6PD* Viangchan) was the most common (83.3%), after that nt 487 G>A (*G6PD* Mahidol) (11.9%), and nt 1360 C>T (*G6PD* Union) (4.8%) (Nantakomol *et al*., 2013).

Thus, *G6PD* deficiency is greatly related with significant neonatal jaundice (Riskin *et al*., 2012). In Malaysia, nine genetic variants have been detected in majority of *G6PD* deficient patients (Wang *et al*., 2008). The total prevalence of *G6PD* deficiency in males is reported as 3.1% in Malaysia, which was more

frequent in Chinese and Malays and less prevalent in Indians (Clarke *et al*., 1997). An individual newborn with *G6PD* deficiency can be additional threatened if there is coexpression with other variants affecting bilirubin conjugation or uptake, and can be in even higher possible of emerging hazardous serum bilirubin levels (Kaplan and Hammerman, 2010).

The *G6PD* mutations found in the present study were nt 1388G>A (60.0%) (20/836 Malay:2.4%, 1/190 Chinese:0.5%), nt 871G>A (17.1%) (5/836 Malay:0.6%, 1/190 Chinese:0.5%), nt 487G>A (14.3%) (5/836 Malay:0.6%), nt 1376G>T (5.7%) (1/836 Malay:0.1%, 1/190 Chinese:0.5%) and nt 1003G>A (2.9%) (1/836 Malay 0.1%). The present study did not find any mutation in the Malaysian Indian neonates. The previous Malaysia study (Ainoon, 2004) reported that the overall prevalence of male *G6PD* deficiency was 4.7% with an ethnic breakdown of 4.6%, 6.0% and 1.3% in the Malays, Chinese and Indians individually.

Variant nt 1388G>A mutation of *G6PD* gene (*G6PD* Kaiping) was 2.4% (17/696) in the SigNH and 0.9% (4/425) in non-SigNH and this variant was detected in Malay and Chinese ethnic groups. In this study, Malay and Chinese have 2.5% and 0.5% respectively to have this mutation. The study by Wang *et al* (2008) found that 1388G>A is a commonly found variant in the Malaysian Chinese and of the 27 newborns with *G6PD* variants detected, three (all female) were heterozygotes and 24 (17 males, 7 females) were homozygotes.

The two nucleotide substitutions of cDNA 1388 (*G6PD* Kaiping) and cDNA 1376 (*G6PD* Canton) have been reported as common mutations among Chinese with *G6PD* deficiency. It is rather interesting that both substitutions are located in exon 12 of the *G6PD* gene (Chiu, 1993). Beutler, 1991 has stated that the more severe form of *G6PD* deficiency is due to mutations clustering near the putative NADP binding site of *G6PD*, whereas milder forms of *G6PD* deficiency are mostly caused by mutations near the amino acid end of the enzyme.Although both cDNA 1388 and 1376 substitutions are not in the immediate vicinity of the putative NADP binding domain, their close proximity to this region may rather the kinetics of *G6PD* in such a way that individuals with either of these two variants would be more susceptible to chemical-induced hemolytic crisis.

This 1388G>A variant mutation was frequently reported in Chinese population (Jiang, 2006) and was commonly detected in Singapore (Sah., 1994, Hamada, 2010) and Vietnam (Matsuoka, 2007) proposing that these mutations spread from China to these states in the former times. Their studies detected *G6PD* Kaiping in the Malaysian Indians but our study did not find this mutation in Indian babies. This 1388G>A variant mutation was more commonly found in Malays in the study whereas the study by Ainoon *et al* (Ainoon, 2004) found that nt 1388G>A was also one of the most frequent mutation in *G6PD*-deficient Malaysian Chinese male neonates (37.5%) but compared to their previous study (Ainoon, 2002), 1388 G>A was not common in Malaysian Malay male neonates (2.3%). Our finding is

also different from Hamada *et al* study (2010) that they found this mutation, and was mainly detected in Singapore Chinese people. The study by Hu (2015) reported that nt G1388A is one of the most frequent mutation in Chinese Hakka people of southern Jiangxi province.

The nt 871G>A mutation of *G6PD* (*G6PD* Viangchan) was 0.6% (4/696) of SigNH and 0.5% (2/425) in non-SigNH and this variant was detected in Malay and Chinese ethnic groups with frequency of 0.6 and 0.5% respectively. This result is consistent with Sulaiman *et al* study (2013) and they found this variant mutation in one of the deficient patients (25%) and this finding was also in agreement with those of previous Malay studies (Normah, 1991, Boo, 1995). The study by Yusoff *et al* (2003) observed that *G6PD* Viangchan heterozygous mutations were found among the Malay people (28.7%). The study by Ainoon *et al* (2002) also reported that *G6PD* Viangchan was found 37.2% in Malay male *G6PD*-deficient newborns and only 0.8% of *G6PD* Viangchan was observed in Malaysian Chinese male *G6PD*-deficient newborns. Moreover, Wang *et al* study (2008) detected *G6PD* Viangchan in Orang Asli people (Aboriginal Malaysians) and this defect is often observed in southeast Asian nations.

G6PD Viangchan was first characterized biochemically in 1988 from a Laotian *G6PD*-deficient patient in Canada (Poon *et al*., 1988). This G6PD variant was categorized as WHO class 2, or severely deficient varant (WHO, 1988). Another study (Yusoff, 2003) stated that *G6PD* Viangchan mutation was detected

in Malay people (28.7%). *G6PD* Viangchan has also been reported to be a common mutation in Laos (Iwai, 2001), Singapore with a frequency of 18.0% (Hamada, 2010) and Thai with a frequency of 54.0% (Nuchprayoon, 2002). From these previous results together with our recent result showed that this variant 871G>A is one of the common mutations in Malaysia and other southeast Asian nations.

Variant nt 487G>A mutation of *G6PD* (*G6PD* Mahidol) was 0.7% (5/696) of SigNH and this variant was detected in Malay neonates only in the study. Our result showed low frequency of this mutation compared with previous studies such as Sulaiman et al study (2013) indicated that 75% of *G6PD* deficiency in Malaysian University community was *G6PD* Mahidol (487G>A) and Ainoon *et al* study (2002) also found 15.1% of *G6PD* deficiency cases had *G6PD* Mahidol in Malaysian Malay male neonates and 1.6% of *G6PD* Mahidol was found in Malaysian Chinese male *G6PD*-deficient newborns.

In many parts of Greater Mekong Subregion (GMS), this Mahidol variant has significant levels of prevalence (Li, 2015). The Mahidol mutation has extended 88-96% prevalence in *G6PD* deficient individuals at the Myanmar-Thai border region (Bancone, 2014, Phompradit, 2011). This defect does not appear to damage the catalytic efficiency of the enzymatic protein but slightly affects protein stability and folding (Huang, 2008). This defect also causes a mild to moderate decline of *G6PD* activity to 5-32% of normal activity in healthy subjects (Panich, 1972), while most individuals with the G487A variants are generally producing no symptoms

with no severe anemia (Tanphaichitr, 1995). *G6PD* Mahidol is a frequent mutation among Thais (Panich, 1972). It was detected in Laotian immigrants in Hawaii (Beutler, 1992) and afterward observed to be a dominant mutation in Myanmar (Iwai, 2001).

The existence of the Viangchan mutation at a high occurrence and the presence of the Mahidol variant in the Malays in the present study is consistent with previous Malay study (Ainoon, 2002) and these seem to reflect a common ancestral origin of the Malays with other southeast Asian peoples precisely Cambodians, Laotians, and Thais. This seem to be an important result in the research of Malaysian ethno-history since the proofs on the origin of the indigenous individuals of Malaysia before the 14th Century have been inadequate (Ainoon, 2002). There are evidences of strong connection between the Neolithic peoples in central and southern Thailand and the early Malaysia inhabitants which is reinforced by the fact that present-day aboriginal population of Malaysia express Mon-Khmer associated (Hall, 1984). Hence, other researchers propose that it is likely that the Malays may have the originated from these Austronesian-speaking population (Andaya, 2001). Malaysia does not belong to the Indochina state, but sharing of the *G6PD* Mahidol and *G6PD* Viangchan with Thais, Laotians, and Cambodians definitely support to the philosophy of genetic drifts through early people movement from the Indochina Peninsular instead of from South China. (Ainoon, 2002). Alteration in genetic heterogeneity of the people and sample size could account for this inconsistency.

Furthermore, nt 1376G>T heterozygous mutation of *G6PD* gene (*G6PD* Canton) was 0.3% (2/696) in SigNH only and this variant was detected in Malay male and Malaysian Chinese male. However, the study by Ainoon (1995) found high frequency of 61.0% of the *G6PD*-deficient Malaysian Chinese male newborns positive for this defect. Their study observed that there were three different forms of electrophoretic mobility in the mutation nt G1376T and this described that varied biochemical variants may share the same point mutation. Their study also observed that all the subjects with the mutation G+T at nucleotide 1376 had neonatal hyperbilirubinemia within the early neonatal period, but none with severe neonatal jaundice. Another study of Wong *et al* (2009) detected that 1376G>T was the most common in Malaysian Chinese. Moreover, nucleotide 1376G>T is one of the two most common alleles associated with *G6PD* deficiency detected by China study groups (Chiu, 1991, Zuo, 1992) and others Southeast Asian countries (Stevens, 1990).

The high incidence of *G6PD* Canton was also observed in Singapore 24.0% (Hamada, 2010). This inconsistent finding may be described by the different population researches and the method used in mutation analysis. The high incidence of cDNA 1376 mutation among Chinese people can explain the obvious alteration in the clinical manifestations between *G6PD*-deficient Chinese and *G6PD*-deficient American blacks (Chiu, 1993). Beutler (1991) has stated the more severe type of *G6PD* deficiency is because of mutations clustering close to the putative NADP binding position of *G6PD*, while milder types of *G6PD* deficiency are generally

caused by mutations adjacent the amino end. Even though cDNA 1376 substitution is not in the instant vicinity of the putative NADP binding domain (Hirono, 1989), the nearby region can change the kinetics of *G6PD* in such mode that patients with this allele would be more susceptible to chemical-induced hemolytic disaster (Zuo, 1992). In contrast, the defects causing to the A^- or A variant among African Americans are situated distant from the putative NADP binding region near the amino end. It is also should be noted that even if the nature of the defects may be a main contributing factor of clinical severity in *G6PD* deficiency, other causes such as environment, nutrition, and extra genetic aetiologies may play a role in the pathogenesis of *G6PD* deficiency (Zu, 1992).

In addition, nt 1003G>A heterozygous mutation of *G6PD* gene (*G6PD* Chatham) was found 0.1% (1/696) in SigNH and this variant was detected in a Malay male. This low frequency finding is consistent with the study by Ainoon (2002, 2004) reported that *G6PD* Chatham variant (1003G>A) was found 2.3% (2 of 86) in Malay newborns and 0.8% (1 of 87) in Malaysian Chinese newborn respectively in their study and this mutation was also observed in Damascus and two Chinese Indians (Vulliamy, 1988, Lie-Injo, 1977). The present finding of low frequency is also in line with Singapore study showed 2.0% (Hamada, 2010). This low frequency mutation in the Malay population may be due to the dissimilar ancestral influences to the current gene pool in multi-ethnic Malaysia or they could have ascended independently, as in numerous other peoples.

Although none of the variant mutations of *G6PD* gene showed significant differences in their frequencies, the recent study can show the association between mutations pattern in significance hyperbilirubinemic Malaysian neonates of main ethnic groups. In our study, 3.1% of neonatal jaundice babies developed *G6PD*-deficiency and from that 82.9% (29/35) were from SigNH group. This finding can highlight *G6PD* deficiency had a higher tendency towards hyperbilirubinemia.

5.3 Conclusion

In conclusion, the current study shows the pattern of frequency of G6PD mutations in SigNH. The most common mutation variant of *G6PD* gene in our study was 1388G>A (*G6PD* Kaiping). Variants *G6PD* gene at nucleotide 1388G>A (*G6PD* Kaiping), nucleotide 871 G>A (*G6PD* Viangchan), nucleotide 1376G>T (*G6PD* Canton) and nucleotide 1003G>A (*G6PD* Chatham) were found to be more commonly detected in SigNH in the main ethnic groups in our study. In addition, co-expressed mutation cases are more associated with SigNH group.

The odds ratios (ORs), adjusted for covariates and logistic regression for variants nt 1388G>A, nt 871 G>A, nt 487G>A, nt 1376G>T and nt 1003G>A of *G6PD* gene were not statistically significant in genotypic distribution between SigNH and non-SigNH. However, our study showed that both genders with mutation were risk of evolving severe hyperbilirubinemia. For females, both homozygous and heterozygous were at risk.

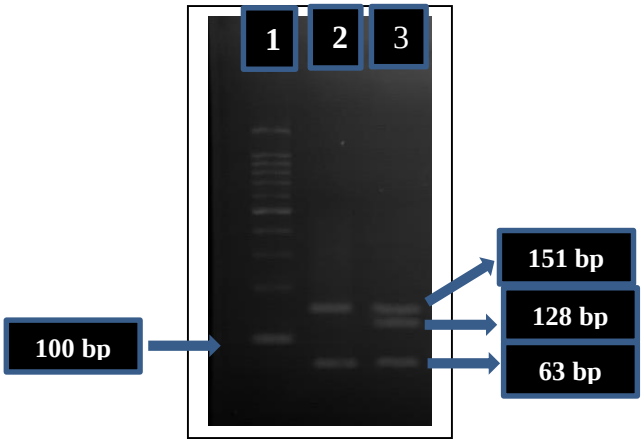
In future, the findings may contribute to the improvement in the frequency of identification of *G6PD* variants in South-east Asia. It could be useful for future prevention and control of *G6PD* deficiency. Once *G6PD* mutation is confirmed, close monitoring, health education regarding triggers and proper follow-up for hemolysis can prevent severe hemolysis, thus decreasing morbidity and mortality in patients with *G6PD* deficiency.

CHAPTER 6

SOLUTE CARRIER ORGANIC ANION TRANSPORTER 2 (*SLCO1B1*) GENE POLYMORPHISMS

6.1 Results

Out of total 1121 subjects studied, 49.3% (553/1121) were found to have one of the two variants of *SLCO1B1* gene mutation.



Lane 1: Ladder 100 bp
Lane 2: AA homozygote
Lane 3: GA heterozygote

Figure 6.1: PCR and restriction pattern of (c.388G>A) *SLCO1B1* gene. Lane 1 is a 100 bp DNA marker. Lane 2 and 3 are restriction fragment pattern of PCR products after digesting with *Taq I* and running in 3% agarose gel

In Figure 6.1, the primary PCR product for 388G>A was 214 bp long and included three *Taq I* sites which resulted in four restriction fragments of 151 bp,

128 bp, 63 bp, and 23 bp. Homozygous AA was represented by two fragments (151 bp and 63 bp). PCR products of heterozygote GA was digested at three restrictions sites and produced four fragments (151 bp, 128 bp, 63 bp, and 23 bp). However, fragment of 23 bp could not be observed clearly due to small fragment size.

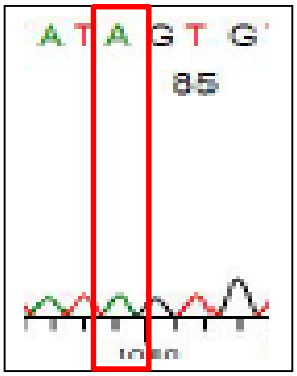
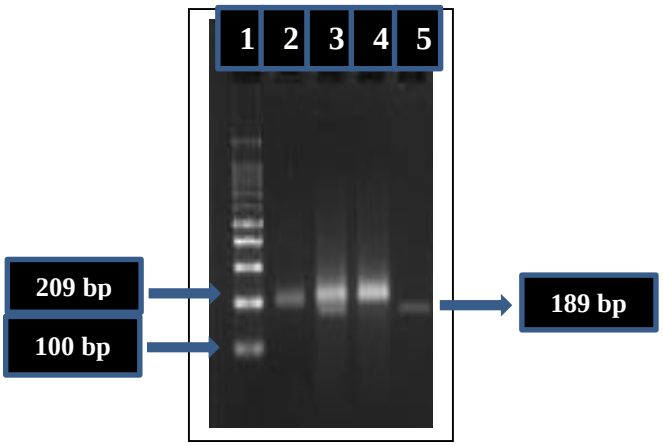


Figure 6.2: Sequence chromatogram of *SLCO1B1* gene variant c.388G>A showing AA homozygous mutation

Figure 6.2 shows sequence chromatogram of *SLCO1B1* gene homozygous variant c.388G>A. The variant c.388G>A mutation of *SLCO1B1* gene found to have 178 heterozygous states (92.7%, 178/192) and 14 homozygous states (2.8%, 14/192) in SigNH group. There were 99 heterozygous cases (96.1%, 99/103) and 4 homozygous states (3.9%, 4/103) in non-SigNH populations.



Lane 1: Ladder 100 bp
Lane 2 and 4: TT homozygote
Lane 3: TC heterozygote
Lane 5: CC homozygote

Figure 6.3: PCR and restriction pattern of (c.521T>C) *SLCO1B1* gene. Lane 1 is a 100 bp DNA marker. Lane 2 to 5 are restriction fragment pattern of PCR products after digesting with *Hha I* and running in 3% agarose gel

In Figure 6.3, the primary PCR product for 521T>C was 227 bp long and included two Hha I sites which resulted in three restriction fragments of 209 bp, 189 bp, and 20 bp. Homozygous TT genotype was represented by one fragment (209 bp) while homozygous CC was represented by two fragments (189 bp and 20 bp). PCR products of heterozygote TC was digested at two restrictions sites and produced three fragments (209 bp, 189 bp, and 20 bp). However, fragment of 20 bp could not be observed clearly due to small fragment size.

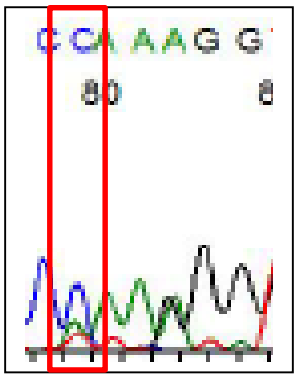


Figure 6.4: Sequence chromatogram of *SLCO1B1* gene variant c.521T>C showing CC homozygous mutation

Figure 6.4 shows sequence chromatogram of *SLCO1B1* gene homozygous variant c.521T>C. The variant c.521T>C mutation of *SLCO1B1* gene found to have 155 heterozygous states (95.1%, 155/163) and 8 homozygous states (4.9%, 8/163) in SigNH group. There were 91 heterozygous cases (95.8%, 91/95) and 4 homozygous states (4.2%, 4/95) in non-SigNH populations.

Genotypic frequency distribution of variants of *SLCO1B1* gene were 388G>A (27.6%, 192/696 in SigNH and 24.2%, 103/425 in non-SigNH) and 521T>C (23.4%, 163/696 in SigNH and 22.3%, 95/425 in non-SigNH) in our study population. Table 6.1 showed the genotypic and allelic distribution of the two variants of *SLCO1B1* mutations in the study. There is no significant difference in the frequencies of genotypic and allele distribution of 388G>A (OR: 1.80, 95% CI = 0.57-5.66, p = 0.28) and 521T>C (OR: 1.17, 95% CI = 0.34-4.01, p = 0.89) mutations.

Table 6.1: Genotypic and allelic frequencies of *SLCO1B1* mutations

Category	*n = 1121*		OR (95% CI)	p-value
	SigNH (TSB≥291 umol/L) (*n* = 696)	Non-SigNH (TSB<291 umol/L) (*n* = 425)		
388G>A				
G/G	504 (61.0%)	322 (39.0%)	1.16 (0.87-1.53)	
G/A	178 (64.3%)	99 (35.7%)	1.80 (0.57-5.66)	0.28
A/A	14 (77.8%)	4 (22.2%)	2.08 (0.67-6.42)	
Alleles				
A	0.148	0.125	1.20 (0.93-1.54)	0.50
521T>C				
T/T	533 (61.8%)	330 (38.2%)	1.05 (0.79-1.41)	
T/C	155 (63.0%)	91 (37.0%)	1.17 (0.34-4.01)	0.89
C/C	8 (66.7%)	4 (33.3%)	1.24 (0.37-4.14)	
Alleles				
C	0.124	0.115	1.06 (0.82-1.38)	0.37

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

The genotypic and gender distribution of *SLCO1B1* mutations in the study are shown in the Table 6.2. There was a slightly higher percentage of *SLCO1B1* mutations in males (53.9%, 298/553) compared to female (46.1%, 255/553) in our study population [(OR: 0.82, 95% CI = 0.63-1.07, p = 0.17) and (OR: 1.08, 95% CI = 0.82-1.43, p = 0.84)].

Table 6.2: Genotypic and gender distribution of *SLCO1B1* mutations

Category	*n = 1121*		OR (95% CI)	p-value
	Male (*N* = 574)	Female (*N* = 574)		
388G>A				
Wild Type	412 (49.9%)	414 (50.1%)	0.82 (0.63-1.07)	0.17
Mutant	162 (54.9%)	133 (45.1%)		
521T>C				
Wild Type	438 (50.8%)	425 (49.2%)	1.08 (0.82-1.43)	0.84
Mutant	136 (52.7%)	122 (47.3%)		

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

The present study found *SLCO1B1* gene mutation in all the ethnic groups but the highest number of mutations was seen in Malaysian Malay (85.8%, 474/553) followed by Malaysian Chinese (9.9%, 55/553) and Malaysian Indian (4.3%, 24/553) neonates in our study population.

The genotypic and ethnic distribution of *SLCO1B1* c.388G>A mutation in the study are shown in Table 6.3. The c.388G>A mutation in the *SLCO1B1* gene was found 27.6% (192/696: 178 heterozygous and 14 homozygous; Malay 30.5%, Chinese 23.6% and Indian 36.8%) in SigNH group and of 24.0% (103/425: 99 heterozygous and 4 homozygous; Malay 26.5%, Chinese 15.9% and Indian 35.3%) in non-SigNH group [(OR: 3.58, 95% CI = 1.70-7.55), (OR: 2.57, 95% CI = 0.70-9.48) and (OR: 1.55, 95% CI = 0.51-4.77)].

Table 6.3: Genotypic and ethnic distribution of *SLCO1B1* c.388G>A mutations

Category	*n = 1121*		OR (95% CI)
	SigNH (TSB≥291 umol/L)	Non-SigNH (TSB<291 umol/L)	
388G>A			
Malay (*n* = 839)	155 (64.0%)	87 (36.0%)	3.58 (1.70-7.55)
Chinese (*n* = 190)	30 (75.0%)	10 (25.0%)	2.57 (0.70-9.48)
Indian (*n* = 36)	7 (53.8%)	6 (46.2%)	1.55 (0.51-4.77)
Others (*n*=56)	0 (0.0%)	0 (0.0%)	

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

The genotypic and ethnic distribution of *SLCO1B1* c.521T>C mutation in the study are shown in Table 6.4. The c.521C>T mutation in the OATP2 gene was found 23.6% (163/696: 155 heterozygous and 8 homozygous; Malay 28.5%, Chinese 8.7% and Others 18.4%) in SigNH group and 22.1% (95/425: 91 heterozygous and 4 homozygous; Malay 26.5%, Chinese 6.3% and Others 19.0%) in non-SigNH group [(OR: 1.62, 95% CI = 0.50-5.25), (OR: 1.57, 95% CI = 0.29-8.42) and (OR: 1.03, 95% CI = 0.29-3.62)].

Table 6.4: Genotypic and ethnic distribution of *SLCO1B1* c.521T>C mutations

Category	*n = 1121*		OR (95% CI)
	SigNH (TSB≥291 umol/L)	**Non-SigNH (TSB<291 umol/L)**	
521T>C			
Malay (*n* = 839)	145 (62.5%)	87 (37.5%)	1.62 (0.50-5.25)
Chinese (*n* = 190)	11 (73.3%)	4 (26.7%)	1.57 (0.29-8.42)
Others (*n* = 59)	7 (63.6%)	4 (36.4%)	1.03 (0.29-3.62)
Indian (*n* = 36)	0 (0.0%)	0 (0.0%)	
Others (n = 56)	0 (0.0%)	0 (0.0%)	

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

6.2 Discussion

Numerous factors are involed in the development of neonatal hyperbilirubinemia, including the *SLCO1B1* gene, which necessitates further investigation as *SLCO1B1* encodes a liver-specific member of the organic anion transporter family. The encoded protein is a tansmembrane receptor that can rapidly and selectively uptake bilirubin into liver hepatocytes (DerDeure, 2008). The results of this study showed that 49.3% (553/1121) the three ethnic groups of neonates carried a *SLCO1B1* mutation and the more common mutation found was c.388G>A (295/553, 53.3%) followed by 521T>C (258/553, 46.7%).

In the recent study, the frequency of mutations of *SLCO1B1* gene variants was higher in SigNH group (64.2%, 355/553) compared to non-SigNH group (35.8%, 198/553). In addition, the frequency of heterozygous state was significantly higher (94.6%, 523/553) than homozygous state (5.4%, 30/553). Moreover,

majority of heterozygous cases were from SigNH (333/523) compared to non-SigNH (190/523). Similarly, frequency of homozygous state was found higher in SigNH (22/30) compared to non-SigNH (8/30). However, the variants c.388G>A and c.521T>C of *SLCO1B1* gene mutations frequencies were not statistically different between SigNH and non-SigNH.

These findings are consistent with Wong *et al* study (2012) and they stated that c.388G>A is the most frequent *SLCO1B1* variant with a prevalence of 79.5% in the Malaysian Chinese followed by 78.2% in the Malaysian Malays and 54.2% in the Malaysian Indians and c.521T>C was found highest frequency in Malaysian Indians (38.0%) followed by Malaysian Malays (22.8%) and Malaysian Chinese (5.5%). The present study finding is also consistent with Cui *et al* (2001) and Huang *et al* study (2004) that they found c.388G>A is common variant mutation in Asian individuals.

The study of Huang *et al*., 2008 suggested that indirect bilirubin may be a substrate for *SLCO1B1*, which is a hepatic sinusoidal transporter that facilitates the liver uptake of a wide range of xenobiotics and endogenous substrates in an ATP-independent manner (Cui *et al*., 2001). It follows that the developmental expression of *SLCO1B1* (Daood and Watchko, 2006) and non-synonymous variants of *SLCO1B1* gene may impact liver bilirubin uptake kinetics and bilirubin metabolism in newborns. *SLCO1B1* allele variant is known to be associated with risk of neonatal

hyperbilirubinemia and coupling of *SLCO1B1* with *UGT1A1* variant alleles further increases the risk (Huang *et al*., 2004).

An *in vitro* expression study demonstrated that *SLCO1B1* 388 G>A variations are consistently associated with reduced transport activity of *SLCO1B1* (Kameyama, 2005). The study of Johnson *et al*., 2009 reported that variants in the *UGT1A1* gene locus showed strongest association with total serum bilirubin in one of them in approximately 9500 white individuals; the only other significant associated found was with the *SLCO1B1* T521C variant. The study by Sanna reported that 4300 white subjects identified a strong association of noncoding *SLCO1B3* SNP with both unconjugated and conjugated bilirubin, in addition to strong effects of *G6PD* and *UGT1A1* and moderate effects of the *SLCO1B1* nt A388G and nt T521C (Sanna, 2009).

The study by Wong (2009) stated that even though the c.388G>A variants was the most common (90.9%) and c.521T>C was the least common of four *SLCO1B1* variants in their study population of the Malaysian newborns, forward logistic regression analysis showed that all variants of *SLCO1B1* gene mutation were not significant risk factors. However, study by Liu (2013) described that *SLCO1B1* 388G>A arisen significantly more commonly in infants with hyperbilirubinemia than in normal subjects (OR:1.50; 95% CI: 1.13-2.00) and there were no significant differences in *SLCO1B1* 521T>C between the case and the control groups (OR:1.00; 95% CI: 0.72-1.40).

The *SLCO1B1*1B* (c.521T>C) haplotype has been associated with increased *OATP1B1* transport activity *in vitro* sin studies performed with bromosulfophthalein and estrone-3-sulfate. The *SLCO1B1* may be a useful therapeutic target for neonatal hyperbilirubinemia, however further studies are needed to explore this hypothesis, and to confirm whether some SLCO1B1 activators could improve the transporting activity of SLCO1B1 gene, which could enhance uptake of bilirubin from blood to bile and decrease serum bilirubin levels (Liu, 2913).

The study by Liu *et al* (2013) have done a meta-analysis with systematic review on the influence of *SLCO1B1* genetic mutations on neonatal jaundice. Findings of the meta-analysis result showed that there was no significant difference in the hazard of neonatal jaundice between *SLCO1B1* 388G>A allele carriers (A/A+G/A) and G/G allele carriers. A significant inter-study heterogeneity was detected (p=0.00). Egger's test delivered no evidence for funnel plot asymmetry in the contrast of the *SLCO1B1* G388A mutation and neonatal jaundice (t=2.12, p=0.07). Furthermore, in the subgroup analyses based on ethnicity, no significant associations were detected in Whites, Thai (Prachukthum., 2009), Asian, Latin American (Watchko, 2009), or Malaysian populations (Wong, 2009). Nevertheless, a significantly higher risk was observed in Chinese newborns.

Various etiologies are included in the formation of neonatal jaundice, together with the *SLCO1B1* gene, which necessitates further investigation. The encoded protein is transmembrane receptor that can selectively and quickly uptake bilirubin into liver cells (DerDeure, 2008). Mutations within the coding area of the *SLCO1B1* gene may consequence in dysfunction of *SLCO1B1* due to abnormal form (Pasanen, 2008). In adult liver disease, mutation in the gene encoding this protein is a main contributing factor of serum bilirubin levels (Huang, 2005). These results showed that defects in the *SLCO1B1* gene are probably related with neonatal jaundice.

6.3 Conclusion

In conclusion, the present study observed the *SLCO1B1* polymorphisms in neonatal hyperbilirubinemia patients. Although the odds ratios (ORs), adjusted for covariates for the variants c.388G>A and c.521T>C of *SLCO1B1* gene were not significantly different in genotypic distribution between SigNH and non-SigNH, the frequencies of variants c.388G>A and c.521T>C of *SLCO1B1* gene were detected higher in SigNH.

Even though the odds ratios (ORs) for the variant c.388G>A of *SLCO1B1* gene showed not statistically significant difference in genotypic distribution between SigNH and non-SigNH, the logistic regression for the variants c.388G>A mutation showed significant predictor value for significant neonatal hyperbilirubinemia (Table 10.1). Further prospective and repeated study is needed

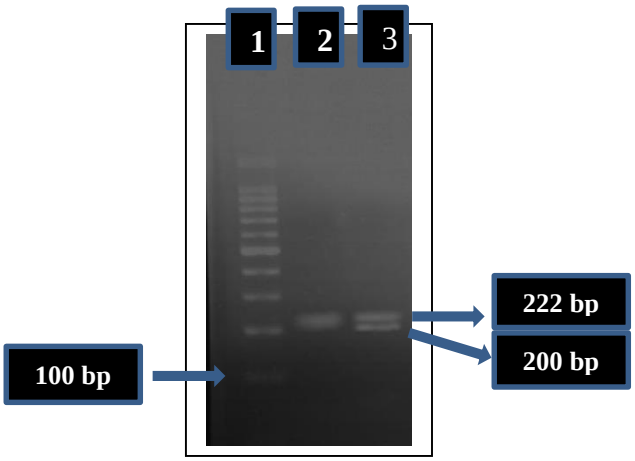
to confirm the association. Future study should involve other variants of *SLCO1B1* and environmental risk factors. It is also necessary to investigate their association with the severity and prognosis of this disease in order to elucidate the genetic pathogenesis of neonatal hyperbilirubinemia as a complex disease.

CHAPTER 7

ALPHA-THALASSAEMIA GENE MUTATION

7.1 Results

Out of total 1121 subjects studied, two cases of heterozygous mutation in the Hb CS gene were detected in SigNH group (0.3%, 2/696).



Lane 1: Ladder 100 bp
Lane 2: TT homozygote
Lane 3: TC heterozygote

Figure 7.1: 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

In Figure 7.1, the primary PCR product for 427T>C was 222 bp long and included two *Taq I* sites which resulted in three restriction fragments of 222 bp, 200 bp, and 22 bp. Homozygous TT was represented by one fragment (222 bp). PCR

products of heterozygote TC was digested at two restrictions sites and produced three fragments (222 bp, 200 bp, and 22 bp). However, fragment of 22 bp could not be observed clearly due to small fragment size.

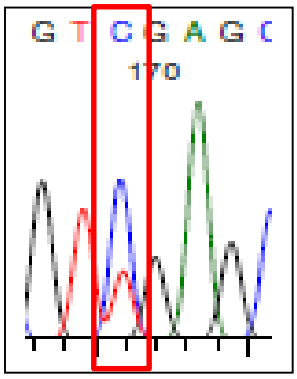


Figure 7.2: Sequence chromatogram of *Hb CS* gene variant 427T>C showing TC heterozygous mutation

Figure 7.2 shows Sequence chromatogram of Hb CS gene heterozygous variant 427T>C.

Table 7.1 showed genotypic, allelic and ethnic distribution of Hb CS (c.427T>C) gene variation. There were two cases of heterozygous mutation in the Hb CS gene with T to C substitution at nucleotide 427 detected in the SigNH group (0.2%: 2/1121) (OR: 4.00, 95% CI = 0.03-591.10, p = 0.53), one of which is a Malay and another is Chinese neonate. No Hb Adana gene mutation was found either in SigNH or in non-SigNH.

Table 7.1: Genotypic, allelic and ethnic distribution of Hb CS (c.427T>C) gene variation

Category	*n = 1121*		OR (95% CI)	p-value
	SigNH (TSB≥291 umol/L) (*n*=696)	Non-SigNH (TSB<291 umol/L) (*n*=425)		
HbCS (c.427T>C)				
Malay (*n* = 836)	1 (100.0%)	0.0%		
Chinese (*n*= 190)	1 (100.0%)	0.0%		
Indian (*n*= 36)	0.0%	0.0%		
Other (*n*= 59)	0.0%	0.0%		
T/T	694 (62.0%)	425 (38.0%)	1.22 (0.11-13.52)	
T/C	2 (100.0%)	0.0%	4.00 (0.03-591.10)	0.53
C/C	0.0%	0.0%		
Alleles				
C	0.001	0	1.23 (0.17-8.70)	0.10

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

7.2 Discussion

Out of 1121 subjects studied, there were two cases of heterozygous mutation in the Hb CS gene detected in the SigNH group. In Malaysia, around 4.5% of Chinese and 2.5% of Malays were carriers of α^0-thalassemia (Southeast Asian (SEA) α-globin gene deletion, $--^{SEA}/\alpha\alpha$) (George, 1998). Studies done on non-deletional α-thalassemia in Malaysia were limited to adults only. The study by Laig et al (Laig *et al*., 1990), the study by Lie-Injo (1972) and the study by Jomoui *et al* (2015) stated that Hb CS is the most common α-globin structural variant in Malaysia (2.24% of Malays, 0.66% of Chinese and 0.16% of Indians) and in other Southeast Asian countries and it ranges between 1-8%. Although there were few young adult or adult case reports on mutation of Hb CS and Hb Adana in Malaysia (Lie-Injo, 1974, George, 2009, Wee, 2009, Azma, 2012), no studies on non-

deletional hemoglobin constant spring (Hb CS) (HbA2: c.427T>C) and hemoglobin Adana (HbA2: c.179G>A) mutations and also these mutations have not been reported in SigNH among Malaysian neonates.

Nainggolan *et al* study reported that hydrops fetalis cases related with nondeletional α-thalassemia (α-thal). The genotypes of the fetuses and their parents were detected by a polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP)-based method and DNA sequencing method to rapidly identify mutations. Two of the fetuses had homozygous α59 (E8) Gly>Asp (α2), also named Hb Adana, and hydrops fetalis. As both parents were carriers of this mutation, the third fetus was also suspected to be homozygous Hb Adena. This study showed that homozygosity for Hb Adena is related with hydrops fetalis in the Indonesian population (Nainggolan *et al*., 2010).

Each mutation precisely alters the termination codon TAA so that instead of the chain terminating, an amino acid is inserted. Read-through of mRNA follows this process that is not normally translated until another "in-phase" stop codon is reached. Therefore, each of these variants has a lengthened α chain. The α-globin mRNA "read-through" that generally is not utilized likely decreases its stability. Numerous nonsense mutations happen, for instance, one in exon 3 of the α_2-globin gene. Lastly, numerous mutations occur that cause α-thalassemia by forming extremely unstable α-globin chains, including hemoglobins Suan Doc, Petah Tikvah, QuongSze, and Evanston (Williams, 2016).

Numerous processing mutations have been observed. For instance, the 5′ splice site of IVS-1 of the α_2-globin gene is included in a pentanucleotide deletion. This mutation involves the invariant GT donor splicing sequence and therefore entirely inactivates the α2 gene. The poly-A addition signal site (AATAAA>AATAAG) and downregulates the α2 gene by interfering with 3′ end processing is involved in a second mutant of this type, detected commonly in the Middle East (Williams, 2016).

Initially, identification of common deletional types of α-thalassemia was planned in the present study. However, due to the difficulty of obtaining successful PCR amplication with deletional type α-thalassemia primers with FTA filter paper, only the site mutational type was able to be determined in the study. Furthermore, in previous reported studies, deletional type α-thalassemia studies were amplified from mainly whole blood samples. The DNA concentration from the FTA card sample was probabaly not adequate for such amplicfication to be done efficiently and there may be some inhibitors which could probably interfere with the PCR reaction as well.

7.3 Conclusin

In conclusion, non-deletional Hb CS mutation was detected in SigNH group in our study population. Although there was very low frequency of mutation detected in our study, this would be the new finding of this mutation in significant

neonatal hyperbilirubinemia in Malaysia. The detection of Hb CS in neonates with significant hyperbilirubinemia may warrant further investigations of its implication among neonatal hyperbilirubinemic patients.

Approaches to the better control and treatment of the thalassemias in poor nations have been reviewed. These contain the development of partnerships between centers in the poor and rich nations for training employees in this field, and, once these are established, for the further evolution of partnerships between those poorer nations where there is information and expertise of the field with those where no facilities or knowledge exist. Deprived of organizations along these lines the thalassemias will remain to happen the worldwide early death of hundreds of thousands of infants (Williams, 2016). Future study should involve deletional types of α-thalassemia variants and environmental risk factors by using whole blood sample.

CHAPTER 8

COEXPRESSION AND DISCUSSIONS

8.1 Results

In the present study, altogether eighteen variants of *UGT1A1*, *G6PD*, *SLCO1B1* and α -thalassemia gene mutations were studied in SigNH and non-SigNH subjects. Out of 18 variants studied, the study detected 14 variants mutations of *UGT1A1*, *G6PD*, *SLCO1B1* and α -thalassemia genes. A total of 357 coexpressed cases were found in both SigNH and non-SigNH. From these, 97.5% (348 of 357) showed 2 variants coexpression and 2.5% (9 of 357) showed 3 variants coexpression.

Coexpression cases of *UGT1A1* promoter A$(TA)_n$TAA and c.-3279T>G mutations in the study are shown in Table 8.1. The coexpression cases were found higher frequency in SigNH (62.5%, 65/104) than non-SigNH subjects (34.8%, 39/104). There was a significant difference in the frequency of coexpression between $(TA)_6$/$(TA)_7$ and c.-3279G/G [OR: 1.01 (95%CI: 0.09-10.66; p = 0.04)].

Table 8.1: Coexpression of *UGT1A1* promoter A(TA)$_n$TAA and c.-3279T>G mutations

Category	*n = 1121*		OR (95% CI)	p-value
	SigNH (TSB≥291 umol/L) (*n*=696)	**Non-SigNH (TSB<291 umol/L) (*n*=425)**		
(TA)6/(TA)7 and c.-3279T/G	27 (48.2%)	29 (51.8%)	0.23 (0.08-0.66)	0.06
(TA)6/(TA)7 and c.-3279G/G	24 (80.0%)	6 (20.0%)	1.01 (0.09-10.66)	0.04*
(TA)7/(TA)7 and c.-3279T/G	4 (80.0%)	1 (20.0%)	1.2 (0.09-15.26)	0.45
(TA)7/(TA)7 and c.-3279G/G	10 (76.9%)	3 (23.1%)	3.58 (0.89-14.41)	0.24

* $p<0.05$

Note: All zero count was continuity corrected with 0.5.

Coexpression cases of *G6PD* and *UGT1A1* mutations in the study are shown in Table 8.2. Majority of coexpressions cases were from SigNH group (6 of 8, 75.0%) than non-SigNH group (2 of 8, 25.0%). There is no significant difference in the frequency of these coexpression cases between SigNH and non-SigNH cases.

Table 8.2: Coexpression of *G6PD* and *UGT1A1* mutations

Category	*n = 1121*		P-value
	SigNH (TSB≥291 umol/L) (*n*=696)	**Non-SigNH (TSB<291 umol/L) (*n*=425)**	
c.1388A/A and (TA)7/(TA)7	1 (100.0%)	0 (0.0%)	1.0
c.487G/A and (TA)6/(TA)7	1 (100.0%)	0 (0.0%)	1.0
c.1388A/A and c.-3279T/G	1 (100.0%)	0 (0.0%)	1.0
c.1388G/A and c.-3279G/G	2 (100.0%)	0 (0.0%)	0.9
c.1388G/A and c.-3279T/G	0 (0.0%)	1 (100.0%)	1.0
c.871G/A and c.-3279G/G	1 (100.0%)	0 (0.0%)	1.0
c.871G/A and c.-3279T/G	0 (0.0%)	1 (100.0%)	1,0

* p<0.05

Coexpression cases of *G6PD* and *SLCO1B1* mutations in the study are shown in Table 8.3. All coexpression cases were from SigNH (100.0%) group. There is also no significant difference in the frequency of these coexpression cases between SigNH and non-SigNH cases.

Table 8.3: Coexpression of *G6PD* and *SLCO1B1* mutations

Category	*n = 1121*		p-value
	SigNH (TSB≥291 umol/L) (*n*=696)	**Non-SigNH (TSB<291 umol/L) (*n*=425)**	
c.871G/A and c.388G/A	1 (100.0%)	0 (0.0%)	1.0
c.1388A/A and c.388G/A	1 (100.0%)	0 (0.0%)	1.0
c.487G/A and c.521T/C	1 (100.0%)	0 (0.0%)	1.0

* p<0.05

The coexpression cases of *SLCO1B1* and *UGT1A1* mutations in the study are shown in Table 8.4. The highest coexpression cases were seen in c.388G/A and c.-3279T/G followed by c.521T/C and c.-3279T/G mutations. However, only c.521T/C and c.3279T/G coexpression showed significance difference in the frequency among coexpression cases with *SLCO1B1* and *UGT1A1* mutations (p = 0.03).

Table 8.4: Coexpression of *SLCO1B1* and *UGT1A1* mutations

Category	*n = 1121*		p-value
	SigNH (TSB≥291 umol/L) (*n*=696)	**Non-SigNH (TSB<291 umol/L) (*n*=425)**	
c.388G/A and c.-3279T/G	81 (54.0%)	69 (46.0%)	0.76
c.388G/A and c.211G/A	4 (100.0%)	0 (0.0%)	0.52
c.388G/A and (TA)6/(TA)7	1 (100.0%)	0 (0.0%)	1.0
c.521T/C and c.-3279T/G	41 (52.6%)	37 (47.4%)	0.03*
c.521T/C and c.211G/A	1 (100.0%)	0 (0.0%)	1.0

* p<0.05

Three coexpression cases of *G6PD* and *UGT1A1* mutations in the study are shown in Table 8.5. All these variants coexpression cases were from SigNH (100.0%) group. There is no significant difference in the frequency of these coexpression cases between SigNH and non-SigNH cases.

Table 8.5: Three variants coexpression of *G6PD* and *UGT1A1* mutations

Category	*n = 1121*		p-value
	SigNH (TSB≥291 umol/L) (*n*=696)	Non-SigNH (TSB<291 umol/L) (*n*=425)	
c.1388G/A, c.-3279T/G, (TA)6/(TA)7	1 (100.0%)	0 (0.0%)	1.0
c.487G/A, c.-3279T/G, (TA)6/(TA)7	1 (100.0%)	0 (0.0%)	1.0

* p<0.05

The three variants coexpression cases of *SLCO1B1* and *UGT1A1* mutations in the study are shown in Table 8.6. Majority of three variants coexpression cases were from SigNH group (6 of 7, 85.7%). However, only one coexpression of c.388G/A, c.-3279T/G, $(TA)_6/(TA)_7$ case was detected in non-SigNH group.

Table 8.6: Three variants coexpression of *SLCO1B1* and *UGT1A1* mutations

Category	*n = 1121*		p-value
	SigNH (TSB≥291 umol/L) (*n*=696)	Non-SigNH (TSB<291 umol/L) (*n*=425)	
c.388G/A, c.-3279T/G, (TA)6/(TA)7	2 (66.7%)	1 (33.3%)	1.0
521T/C, c.-3279T/G, (TA)6/(TA)7	4 (100.0%)	0 (0.0%)	0.84

* p<0.05

8.2 Discussion

A total of 357 coexpressed cases were found in both SigNH and non-SigNH. From these, 97.5% (348 of 357) showed 2 variants coexpression and 2.5% (9 of

357) showed 3 variants coexpression. Our study did not find any case subjects coexpressed >3 polymorphisms.

The allelic frequency of the *UGT1A1* $(TA)_7$ promoter mutation showed significant difference between SigNH (14.4%) and non-SigNH (9.9%) subjects. Our study observed coexpressed cases with *UGT1A1* variant c.-3279T>G mutation with mutation in the TA promoter region $A(TA)_7TAA$. It has been reported that most Gilbert's syndrome patients with A(TA)7TAA homozygous were also homozygous for c.-3279T>G (Mauro et al., 2004, Costa et al., 2005, Ferraris et al., 2006) and this finding suggests a synergistic effect of c.-3279T>G and A(TA)7TAA on transcription activity (Morioka et al., 2010).

UGT1A1 $(TA)_7$ promoter polymorphism and *UGT1A1* nt c.-3279T>G were coexpressed frequently and was detected in SigNH subjects (62.5% 65/104) and non-SigNH subjects (37.5%, 39/104). Of interest, >2 polymorphisms coexpression cases were *UGT1A1* $(TA)_7$ promoter polymorphism and *UGT1A1* nt c.-3279T>G together with other variants such as *G6PD* nt 1388G>A or *G6PD* nt 487G>A or *SLCO1B1* nt 388G>A or *SLCO1B1* nt 521 T>C variations. In addition, *UGT1A1* nt c.-3279T>G also coexpressed with *G6PD* nt 1388G>A, *G6PD* nt 871G>A, *SLCO1B1* nt 388G>A, and *SLCO1B1* nt 521 T>C. Majority of coexpressed cases with *UGT1A1* were found in Malay ethnic group.

Thirty-five subjects carried *G6PD* mutation of different variants and overall distribution differ significantly between SigNH and non-SigNH subjects (82.9% vs 17.1%). Moreover, *G6PD* mutation subjects who coexpressed with other variants were detected more often among SigNH (81.8%, 9/11) than non-SigNH (8.2%, 2/11). Moreover, five cases of *G6PD* nt 1388G>A coexpressed with *UGT1A1* nt c.-3279T>G and/or *UGT1A1* $(TA)_7$ promoter or *SLCO1B1* nt 388G>A mutation and only one case was found from non-SigNH group. From these, only one case of *G6PD* nt 1388G>A from SigNH group showed 3 polymorphisms with *UGT1A1* nt c.-3279T>G and *UGT1A1* $(TA)_7$ promoter. In addition, 2 cases of *G6PD* nt 871G>A coexpressed with *UGT1A1* nt c.-3279T>G and from these, one case was from SigNH group and another one case was from non-SigNH group. Two cases from SigNH with *G6PD* nt 487G>A coexpressed with either *UGT1A1* $(TA)_7$ promoter repeat or *SLCO1B1* nt 521T>C. All these coexpressed cases of G6PD mutations were detected in Malay male neonates.

The *SLCO1B1* gene variant allelic frequencies did not differ significantly between SigNH and non-SigNH subjects (53.9% vs 46.1%) in the study cohort. However, *SLCO1B1* nt 388G>A coexpressed with *UGT1A1* c.-3279T>G c were found in SigNH (54%) and non-SigNH subjects (46%). In addition, *SLCO1B1* nt 521T>C coexpressed with *UGT1A1* nt c.-3279T>G cases were found significantly higher SigNH subjects than SigNH subjects ($p = 0.03$). There were seven cases of 3 polymorphisms coexpression (3 cases with *SLCO1B1* nt 388G>A and 4 cases of

SLCO1B1 nt 521T>C) with *UGT1A1* c.-3279T>G together with *UGT1A1* $(TA)_7$ promoter. All these coexpressed cases were observed from Malay ethnicity.

The pathogenesis of significant neonatal hyperbilirubinemia frequently is multifactorial (Huang, 2004, Newman, 2000). In the present study, ≥2 genes coexpression cases were more commonly found in SigNH than non-SigNH subjects (57.3% vs 42.7%) and this finding is also consistent with previous studies (Huang, 2004, Newman, 2000). The possibility that this multifactorial nature may include several genetic and environmental contributors concurrently (Watchko, 2009). Expression of variant genes involved in bilirubin metabolism contributes to the multifactorial nature of neonatal jaundice (Kaplan, 1997, Huang, 2002, Kaplan, 2007). Moreover, the recent study found *UGT1A1* variant genotypes coexpressed with *SLCO1B1* polymorphisms were not seen more frequently on SigNH subjects. This finding is also consistent with Huang *et al* study (Huang, 2004) and another Huang *et al* study (Huang, 2005).

In Korea, Kang *et al* study reported that approximately 1000 Koreans subjects were identified; only variants in *SLCO1B3* and *UGT1A1* loci were associated with total serum bilirubin concentrations at the genome-wide significance level and significant associations were found at S*LCO1B3* and *UGT1A1* [(rs 2417940, p=1.03x10 (-17)) and (rs 11891311, p=4.78x10 (-148))] (Kang *et al*., 2010).

Findings on genetic variants observed at increased frequency included nine SigNH subjects carrying the *G6PD* mutation coupled with coexpressed *UGT1A1* or *SLCO1B1* coding sequence variants and two non-SigNH subjects coexpressed with *UGT1A1* variants. This finding also in line with previous Bosma *et al* study (Bosma, 1995). Moreover, Kaplan *et al* studies (Kaplan, 1997, Kaplan, 2007) reported cases with *UGT1A1* variant coexpression with *G6PD* mutations. *UGT1A1* promoter or coding sequence variant coexpression with *G6PD* mutations limits *UGT1A1* expression, and therefore bilirubin conjugation, and may be a precondition for increased hyperbilirubinemia risk among *G6PD*-deficient patients (Kaplan, 2007). The greater prevalence of this co-expression pattern in SigNH subjects versus non-SigNH subjects is consistent with Kaplan *et al* study (Kaplan, 2007, Kaplan, 1997) and this finding can support a genetic interaction between *UGT1A1* and *G6PD* variants in the pathogenesis of hyperbilirubinemia risk.

The data from the current study found only three coexpression cases of *SLCO1B1* variant with *G6PD* mutation in SigNH group and this would be predicted to enhance their hyperbilirubinemia risk (Huang, 2004). Indeed, homozygosity for the *SLCO1B1* G388A variant significantly increased the risk for a TSB level of >20mg/dL (OR:3.02 [95% confidence interval: 1.30-6.99]) in one report (Huang, 2004). Kaplan et al study (Kaplan, 2004) found 8 of the 18 newborns (44%) in their cohort who carried a *G6PDA*- mutation were case subjects (TSB levels of >95th percentile) who coexpressed *SLCO1B1* and/or *UGT1A1*gene variants, a prevalence

of significant hyperbilirubinemia that is higher than the 21.9% reported for black male individuals with biochemical evidence of *G6PD* deficiency.

In the study, frequent coexpression cases of *UGT1A1* nt c.-3279T>G with *UGT1A1* $(TA)_7$ promoter region were found. Hypomorphic *UGT1A1* promoter sequence polymorphisms including variant c.-3279T>G and TATA box variant *UGT1A1**28 are also found in East Asian individuals (Ramirez, 2010), even though typically at lower allele frequencies than *UGT1A1**6, but their coexpression with *UGT1A1**6 and other *UGT1A1* coding sequence variants to form compound heterozygous genotypes is detected in 6.9% of Chinese newborns (Zhou, 2009).

Coexpression of *UGT1A1* nt c.-3279T>G with *UGT1A1* $(TA)_7$ promoter variant alleles are common and every hyperbilirubinemic (>95th percentile on the Bhutani nomogram) African American newborns in the cohort who was homozygous for (TA)7 was homozygous for T-3279G as well (Watchko, 2009), that is, in the background of a Gilbert genotype, T-3279G and (TA)7 were in linkage disequilibrium. The coupling of hypomorphic *UGT1A1* promoter and coding sequence variants would be expected to decrease *UGT1A1* isoenzyme action further and thus enhance neonatal jaundice. In addition, *UGT1A1* c.-3279T>G polymorphism, a variant itself associated with decreased *UGT1A1* expression (Sugatani, 2002), may contribute an icterogenic consequence.

Coexpression of *UGT1A1* variants with *SLCO1B1* or *G6PD* mutation cases were observed in our study population. Lin *et al* studied in adult US people (Lin., 2008) stated that nearly 50% of individuals who carried the *G6PDA*- mutation coexpressed both (i) the $(TA)_7$ repeat *UGT1A1* promoter variant on ≥1 allele and (ii) homozygosity for either the A1463C or G388A *SLCO1B1* polymorphism. The individual contributions of *SLCO1B1* and *UGT1A1* variants expression to hyperbilirubinemia risk in infants who carry a *G6PD* mutation are not clear. Either *SLCO1B1* or *UGT1A1* variant expression can contribute to hyperbilirubinemia risk in infants (Huang, 2004, Kaplan, 2007, Kaplan, 1997). The second mode of hyperbilirubinemia presentation in *G6PD*-deficient newborns couple low-grade hemolysis with genetic polymorphisms of the *UGT1A1* gene that lower *UGT1A1* expression and thus affect hepatic bilirubin conjugation (Watchko, 2012). A dose-dependent genetic interaction between the *G6PD* deficiency and *UGT1A1**28 promoter variant increases neonatal jaundice (Kaplan, 1997, Herschel, 2002).

In a similar manner, homozygous carriage of the *UGT1A1* nt 211G>A Gilbert genotype predominant in East Asian peoples coexpressed with *G6PD* is reported to increase neonatal hyperbilirubinemia risk (Huang, 2002). On occasion, *G6PD*-deficient patients with Gilbert syndrome may experience an acute hemolytic event with potentially devastating results as would be predicted in the coupling of a marked unconjugated bilirubin production secondary to severe hemolysis with a decreased bilirubin conjugating capacity secondary to low *UGT1A1* enzyme action (Watchko, 2010, Zangen, 2009). Gene polymorphisms of *SLCO1B1*, a bilirubin

transporter (Cui., 2001, Briz., 2000) situated at the sinusoidal membrane of hepatocytes, that is, in the blood-hepatocyte interface, have also been described in association with *G6PD* African A- (Lin, 2008) and may predispose to neonatal jaundice by limiting hepatic bilirubin uptake and thus hepatic bilirubin clearance (Cui, 2001). Indeed, of newborns who carry *G6PD* African A-, those with a TSB>95th percentile on the Bhutani nomogram more often were homozygous for the nonsynonymous *SLCO1B1* G388A polymorphism than those who carried *G6PD* African A- with a TSB<40th percentile (Watchko, 2009).

Uridinediphosphate glucuronosyltransferase 1A1 (*UGT1A1*) expression has been limited by coexpression with promoter area of *UGT1A1* or *G6PD* mutations at coding sequence variant limits, and therefore conjugation of bilirubin, and can be a prerequisite for increased jaundice risk in *G6PD* deficiency individuals. The greater occurrence of this coexpression form in control individuals versus jaundiced individuals is consistent with Kaplan et al finding (Kaplan *et al*., 2007) of a gene interaction between *G6PD* and *UGT1A1* variants in neonatal hyperbilirubinemia threat (Watchko *et al*., 2009).

In addition, a significantly increased risk of hyperbilirubinemia in infants who coexpressed *SLCO1B1* and *UGT1A1* variants, compared with those who expressed *SLCO1B1* or *UGT1A1* polymorphisms alone (Huang, 2004), suggests a synergistic result. The coexpression cases of *UGT1A1* promoter variants and *SLCO1B1* G388A variant were found in SigHN (54.3%) and non-SigNH (45.7%)

subjects. The previous studies (Beutler, 1998, Tirona, 2001, Ho, 2007) reported that the allelic frequencies of *UGT1A1* promoter variants and the *SLCO1B1* nt 388G>A variant are high in patients of African origin, that is *UGT1A1* (TA)7 at 42.6%, *UGT1A1* (TA)8 at 6.9% (Beutler, 1998), and *SLCO1B1* G388A at 74.0% to 77.0% (Tirona, 2001, Ho, 2007). Coupling of iatrogenic *UGT1A1* and *SLCO1B1* variants is reported to augment neonatal hyperbilirubinemia risk in Taiwanese neonates, that further increased risk when the baby is also exclusively breastfed (Huang, 2004).

In future, a number of likely reasons why genetic variants coexpression were more prevalent among SigNH subjects than non-SigNH subjects can be hypothesized. A more comprehensive study is necessary. Due to strong evidence in the literature, genetic polymorphism still should be considered in clinical management, especially in those neonates who have existing risk factors, in order for early intervention to take place and to prevent further neurotoxicity. Bilirubin conjugation genetics awareness and the current abilities to identify genetic mutations of bilirubin conjugation may be useful assistants to the AAP parameters for identifying neonates at risk of developing severe jaundice. Any evaluation of neonatal jaundice, genetic or otherwise, will necessarily include clinical parameters usually evaluated in jaundiced neonates.

8.3 Conclusion

In conclusion, coexpression of 2 genes has not significantly difference between SigNH and non-SigNH babies (210 vs 147), however, coexpression of >2

gene mutations were detected higher in SigNH subjects (88.9%) whereas only one non-SigNH subject demonstrated presence of >2 gene coexpression (11.1%) in our study. In addition, coexpression of *SLCO1B1* variant c.521T>C and *UGT1A1* variant c.-3279T>G mutations showed significant difference in their frequencies between SigNH and non-SigNH group ($p = 0.03$). Moreover, coexpression of heterozygous $(TA)_6/(TA)_7$ and homozygous c.-3279G/G was also risk for significant neonatal hyperbilirubinemia.

CHAPTER 9

FINAL CONCLUSIONS AND FURTHER WORKS

The present study demonstrates that multiple genetic risk factors modulate hyperbilirubinemia risk in newborns. Genotypic distribution and logistic regression analysis of *UGT1A1, G6PD,* alpha-thalassemia and *SLCO1B1* mutations in the study are summarized in Table 9.1. Promoter A(TA)nTAA, variant 211G>A of *UGT1A1* gene and *SLCO1B1* 388G>A variants showed significance risk for SigNH group ($p = <0.05$). However, hetyerozygous c.-3279T>G variant of *UGT1A1* gene showed significantly higher in the frequency in non-SigNH group ($p = 0.001$), thus this allele may be a potential protective factor of such disease. The other gene variants carried no significant risks.

Table 9.1: Genotypic distribution of *UGT1A1, G6PD,* alpha-thalassaemia and *SLCO1B1* mutations in the study

Category	*n = 1121*		p-value
	Wild Type (n)	Mutant Type (n)	
UGT1A1			
211G>A	1096	25	0.04*
686C>A	1112	9	0.90
1091C>T	1118	3	0.10
1456T>G	1111	10	0.41
c.-3279T>G	517	604	0.001*
Promoter A$(TA)_n$TAA	979	142	0.001*
G6PD			
1388 G>A	1100	21	0.11
871 G>A	1115	6	0.76
487G>A	1116	5	0.10
1376G>T	1119	2	0.10
1003G>A	1120	1	1.00
Alpha-Thalassemia			
HbCS (c.427T>C)	1119	2	0.10
SLCO1B1			
388G>A	826	295	0.05*
521T>C	863	258	0.37

* $p<0.05$

Risk assessment of gene variants *UGT1A1* nt 211G>A, promoter A(TA)nTAA and *SLCO1B1* nt 388G>A for significant neonatal hyperbilirubinemia by log-binomial analysis were shown in Table 9.2. This analysis demonstrated that greatest risks of significant hyperbilirubinemia in infants with *UGT1A1* nt 211G>A (RR = 1.431; 95% CI = 1.23-1.67, p = 0.01) followed by promoter A(TA)nTAA of *UGT1A1* gene (RR = 1.143; 95% CI = 1.02-1.29, p = 0.05).

Table 9.2: Risk assessment of gene variants for significant neonatal hyperbilirubinemia by log-binomial analysis (n=1121)

Gene variant	Relative risk	95% CI	p-value
UGT1A1 211G>A	1.431	1.23-1.67	0.01*
UGT1A1 Promoter A(TA)$_n$TAA	1.143	1.02-1.29	0.05*
SLCO1B1 388G>A	1.067	0.97-1.18	0.22

* $p<0.05$

There were some limitations in this study, Firstly, there were lesser number of recruited samples for some ethnic groups such as Malaysian Indian and Malaysian Chinese neonates. Secondly, various other factors may also have contributed to neonatal hyperbilirubinemia, such as environmental factors, which were not included in the recent study. Neonatal hyperbilirubinemia is a clinical manifestation resulting from an extensive range of conditions and although many important associated conditions like prematurity have been excluded by inclusion and exclusion criteria in the study.

There are many other possible aetiologies underlying neonatal jaundice which has to considered in interpretation of association between genetic mutations and neonatal hyperbilirubinemia as multivariate analysis could not be done. Moreover, this study was conducted and based in a single centre so it may not represent the whole population of Malaysia. However, the present study can show

the molecular mutation patterns as quite a substantial number of cases were recruited and analysed.

In conclusion, even though the mutations of *SLCO1B1*, *UGT1A1*, and *G6PD* in neonates may not be fully studied as genetic contribution, a rising literature indicates the important modulatory role of genetic variation across bilirubin metabolism genes can have on hyperbilirubinemia risk in neonates. There are many genetic disorders that are associated with hyperbilirubinemia. For many populations, especially in South and Southeast Asia, further research is needed to elucidate the genetic disorders underlying neonatal jaundice. This suggests there may be some more genetic factors that we have not tested for. Furthermore, genetic factors contribute to neonatal hyperbilirubinemia even in cases where aetiology is obvious. It is generally assumed that genetic factors have more significant role in the causation of idiopathic SigNH.

In addition, the recent study can show the mutation patterns and risks associated with significant neonatal hyperbilirubinemia as this SigNH condition is very important transitional period change to sever (TSB≥342 umol/L) and extreme (TSB≥428 umol/L) neonatal hyperbilirubinemia, and at these stages, very high risk of complications. To the best of our knowledge, this is the first investigation of these four genes (*UGT1A1, G6PD, SLCO1B1,* and alpha-thalassemia) in the study of significant neonatal hyperbilirubinemia in the main ethnic groups of Malaysia in a Malaysian hospital.

The recent study report can help in guiding the diagnosis, clinical management and genotype screening in neonatal hyperbilirubinemia. Moreover, this study also can help in a better predictive assessment on which newborns are at greater risk and it will benefit caretakers in hospitalization and postdischarge outpatient follow-up and assessment. Identification of multiple mutations in patients could therefore be used predictively to identify patients at risk of severe disease.

It is essential that a newborn's jaundice be monitored closely by health care professionals. Health education of the population at risk, especially pregnant women, and early referral at primary health care level will reduce the burden of significant neonatal jaundice. Future studies will further explain the interactions among multiple bilirubin metabolism gene loci, other genes, and non-genetic factors to neonatal jaundice, and will explore their possible epigenetic modification. Rapid and accurate screening systems for these genetic disorders should be established for the proper management of neonatal hyperbilirubinemia so that brain damage can be prevented.

Moreover, this study results would be mostly useful in contributing to the neonatal hyperbilirubinemia pathology, that facilitating early detection and management before complication. In addition, based on risk assessment, it can provide early and focused follow-up and also can perform a systematic management

befor discharge. In future, the accuracy of prediction can be improved by using more larger size of data. Further srudies could be carried out such as genotype-phenotype correlation of neonatal hyperbilirubinemia including more data inputs such as phenotypes of hyperbilirubinemic neonates and environmental factors.

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APPENDIX A

ETHIC APPROVAL

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(Medical Research & Ethics Committee)
KEMENTERIAN KESIHATAN MALAYSIA
d/a Institut Pengurusan Kesihatan
Jalan Rumah Sakit, Bangsar
59000 Kuala Lumpur

Tel. : 03 2282 9082/03 2282 9085
03 2287 4032/03 2282 0491
Faks : 03 2287 4030

Ruj. Kami : ( ) dlm.KKM/NIHSEC/ P14-391
Tarikh : 16 Julai 2014

Dr Chee Siok Chiong
Jabatan Pediatrik
Hospital Selayang

Prof Boo Nem Yun
Fakulti Perubatan & Sains Kesihatan
Universiti Tunku Abdul Rahman

Tuan/ Puan,

NMRR-14-225-19651
Risk factors associated with severe hyperbilirubinemia in normal term infants in Malaysia

Dengan hormatnya perkara di atas adalah dirujuk.

2. Bersama dengan surat ini dilampirkan surat kelulusan saintifik dan etika bagi projek ini. Segala rekod dan data subjek adalah **SULIT** dan hanya digunakan untuk tujuan kajian ini dan semua isu serta prosedur mengenai ***data confidentiality*** mesti dipatuhi. Kebenaran daripada **Pengarah Hospital** di mana kajian akan dijalankan mesti diperolehi terlebih dahulu sebelum kajian dijalankan. Tuan/puan perlu akur dan mematuhi keputusan tersebut.

3. Penyelidik- penyelidik bersama yang terlibat:
- Dr Anita Kaur Ahluwalia
- Dr Maslina Mohamed

4. Adalah dimaklumkan bahawa kelulusan ini adalah sah sehingga **16 Julai 2015**. Tuan/ Puan perlu menghantar *'Continuing Review Form'* selewat-lewatnya 2 bulan sebelum tamat tempoh kelulusan ini bagi memperbaharui kelulusan etika. Pihak tuan/puan juga perlu mengemukakan laporan tamat kajian dan juga laporan mengenai *"All adverse events, both serious and unexpected"* kepada Jawatankuasa Etika & Penyelidikan Perubatan, KKM jika berkenaan. Borang-borang berkaitan boleh dimuat turun daripada laman web MREC (http://www.nih.gov.my/mrec).

Sekian terima kasih.

BERKHIDMAT UNTUK NEGARA

Saya yang menurut perintah,

(DATO' DR CHANG KIAN MENG)
Pengerusi
Jawatankuasa Etika & Penyelidikan Perubatan
Kementerian Kesihatan Malaysia

JAWATANKUASA ETIKA & PENYELIDIKAN PERUBATAN
(Medical Research & Ethics Committee)
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Date : 16 July 2014

NMRR-14-225-19651
Risk factors associated with severe hyperbilirubinemia in normal term infants in Malaysia

Principal Investigator
Dr Chee Siok Chiong
Jabatan Pediatrik
Hospital Selayang

Prof Boo Nem Yun
Fakulti Perubatan & Sains Kesihatan
Universiti Tunku Abdul Rahman

Documents received and reviewed with reference to the above study:
1. Study Proposal, Version 2, dated 14-07-2014
2. Patient information sheet (English) & Informed Consent Form (English) Version 2, dated 12-07-2014
3. Data Collection form
4. Curriculum vitae of investigators

The Medical Research & Ethics Committee, Ministry of Health Malaysia operates in accordance to the International Conference of Harmonization Good Clinical Practice Guidelines.

Project Sites : Hospital Selayang

Please be informed that this approval is only valid until **16th July 2015**. To renew the approval, a completed *'Continuing Review Form'* should to be submitted to MREC at least 2 months before the expiry for the extension of the approval. You are also required to submit a study completion report upon completion of this study and report on all serious and unexpected adverse events to the Medical Research & Ethics Committee if relevant .These forms can be downloaded from the MREC web site (http://www.nih.gov.my/mrec).

Decision by Medical Research & Ethics Committee:
(√) Approved
( ) Disapproved

Date of Decision: 16 July 2014

(DATO' DR CHANG KIAN MENG)
Pengerusi
Jawatankuasa Etika & Penyelidikan
Perubatan
Kementerian Kesihatan Malaysia

UNIVERSITI TUNKU ABDUL RAHMAN

Wholly Owned by UTAR Education Foundation (Company No. 578227-M)

Re: U/SERC/10/2014

29 April 2014

Emeritus Professor Boo Nem Yun
Department of Population Medicine
Faculty of Medicine and Health Sciences
Universiti Tunku Abdul Rahman
Jalan Sungai Long
Bandar Sungai Long
43000 Kajang
Selangor

Dear Professor Boo,

Ethical Approval For Research Project/Protocol

We refer to your application dated 5 March 2014 for ethical approval for your research project which was reviewed by the UTAR Scientific and Ethical Review Committee (SERC) at its meeting on 9 April 2014. We are pleased to inform you that your application has been approved.

The details of your research project are as follows:

Research Title	Risk Factors Associated with Severe Hyperbilirubinemia in Normal Term Infants in Malaysia
Investigator(s)	Emeritus Prof Boo Nem Yun (PI) Dr Alan Ong Han Kiat Dr Shwe Sin Dr Gary Low Kim Kuan Dr Chee Seok Chiong (Selayang Hosp) Dr Anita Kaur Ahluwalia (Selayang Hosp) Dr Maslina Mohamed (Selayang Hosp)
Research Area	Medical and Health Sciences
Research Location	Selayang Hospital & FMHS
No of Participants	1,100 infants
Research Costs	Self-funded
Approval Validity	2014 – 2015

The conduct of this research is subject to the following:

(1) The participants'/legal guardian's informed consent be obtained prior to the commencement of the research.
(2) Confidentiality of participants' personal data must be maintained; and

(3) Compliance with procedures set out in related policies of UTAR such as the UTAR Research Ethics and Code of Conduct, Code of Practice for Research Involving Humans and other related policies/guidelines.

Should personal data of participants be collected, please have the participants/legal guardian in the research signed the attached Personal Data Protection Statement for your records.

The University wishes you all the best in your research.

Thank you.

Yours sincerely,

Professor Dr Lee Sze Wei
Chairman
UTAR Scientific and Ethical Review Committee

c.c Dean, Faculty of Medicine and Health Sciences
Director, Institute of Postgraduate Studies and Research

APPENDIX B

PUBLICATIONS

Journal Publications:

Shwe Sin, Ong Han Kiat, Nem Yun Boo, Seok Chiong Chee, Maslina Mohamed, Michelle Ling Min Min, Anita Kaur Ahluwalia. Identification of Haemoglobin Constant Spring (HbCS) (HbA2: c.427T>C) and Haemoglobin Adana (HbA2: c.179G>A) in Significant Neonatal Hyperbilirubinemia of the Main Ethnic Groups in a Malaysian Hospital. *The Malaysian Journal of Pathology* 2020 (To be submitted).

Nem Yun Boo, **Shwe Sin**, Ong Han Kiat, Seok Chiong Chee, Maslina Mohamed, Michelle Ling Min Min, Anita Kaur Ahluwalia. Genetic factors, age when first TSB was measured and age of admission were risk factors associated with severe hyperbilirubinemia in jaundiced term neonates admitted for phototherapy. *Journal of Tropical Pediatrics* 2020 (Submitted).

Presentations in Conferences - Poster:

Shwe Sin, Alan Ong Han Kiat, Boo Nem Yun, Chee Seok Chiong, A Kaur, M Mohamad, Michelle Ling, 2016. UDP-Glucuronosyl transferase 1A1 (*UGT1A1*) Gene Polymorphisms in Severe Neonatal Hyperbilirubinemia of the Main Ethnic

Groups in a Malaysian Hospital. Malaysian Society of Haematology 13th MSH Annual Scientific Meeting, 14th–16th April 2016, Shangri-La Hotel, Kuala Lumpur.

Shwe Sin, Alan Ong Han Kiat, Boo Nem Yun, Chee Seok Chiong, A Kaur, M Mohamad, Michelle Ling, 2017. UDP-glucuronosyl transferase 1A1 (*UGT1A1*) Gene Polymorphisms in Malaysian Indians presenting with Significant Neonatal Hyperbilirubinemia in a Malaysian Hospital. Inaugural FMHS Scientific Meeting 2017, 25th–27th May 2017, Universiti Tunku Abdul Rahman, Kuala Lumpur.

Shwe Sin, Alan Ong Han Kiat, Boo Nem Yun, Chee Seok Chiong, A Kaur, M Mohamad, Michelle Ling, 2018. Molecular Characterisation of Haemoglobin Constant Spring (HbA_2 c.427T>C) and Haemoglobin Adana (HbA_2 c.179G>A) in Significant Neonatal Hyperbilirubinemia of the Main Ethnic Groups in a Malaysian Hospital. 10th Conference of the Society of Pathology 2018, 13th January 2018, Sky Star Hotel, Yangon.

Shwe Sin, Alan Ong Han Kiat, Boo Nem Yun, Chee Seok Chiong, A Kaur, M Mohamad, Michelle Ling, 2018. Solute Carrier Organic Anion Transporter Family Membrane 1B1 (*SLCO1B1)* Gene Polymorphisms in Malaysian Newborns presenting with Significant Neonatal Hyperbilirubinemia in a Malaysian Hospital. The Belt and Road International Conference on Thalassemia: Advancing Knowledge in Thalassemia, 31st October to 2nd November 2018, Nanning, Guangxi, P.R. China.