SCREENING OF *UGT1A1* GENE AND GENOTYPE-PHENOTYPE CORRELATIONSHIP IN NEONATAL JAUNDICE FROM A SAMPLE OF NEWBORNS IN KELANTAN

by

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PENYARINGAN *UGT1A1* GEN DAN HUBUNGKAIT GENOTIP-PENOTIPIK DALAM PENYAKIT KUNING DARIPADA SAMPLE BAYI BARU LAHIR DI KELANTAN

oleh

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Tesis yang diserahkan untuk
memenuhi keperluan bagi
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LIST OF ABBREVATIONS

µg/µl	:	Microgram per micro litre
µl	:	Microliter
µM	:	Micro mol
µmol/L	:	Micro mol per litre
1 TB	:	1 Timur Belakang
A	:	Adenine
A_{260}/A_{280}	:	Ratio of 260 absorbance/ 280 absorbance
ABO	:	Types of blood group: A, B, O and AB
ACN	:	Acetonitrile
BBB	:	Blood brain barrier
BC	:	Conjugated bilirubin/ direct bilirubin
bp	:	Base pair
BRC1/BRC2	:	Breast cancer type I or II
BU	:	Unconjugated bilirubin molecule/ indirect bilirubin
Buffer AE	:	Elution buffer
Buffer AW	:	Wash buffer
Buffer BL	:	Lyses buffer
Buffer EB	:	Elution buffer
Buffer PB	:	Purification buffer
B-UGT	:	Bilirubin uridine diphosphate-glucuronosyltransferase
C	:	Cytosine
c.	:	Coding number
CACNL1A4	:	Calcium channel gene, voltage-dependent, P/Q type alpha 1A
cDNA	:	Complementary DNA

CGH	:	Comparative genomic hybridization
CN	:	Crigler Najjar syndrome
CNI	:	Crigler Najjar syndrome type I
CNII	:	Crigler Najjar syndrome type II
CNS	:	Central nervous system
COL2A1	:	Collagen, type II, alpha 1
dATP	:	Deoxyadenine triphosphate
dCTP	:	Deoxycytosine triphosphate
ddH_2O	:	Double distil water
ddNTP	:	Modified nucleotides
DHPLC	:	Denaturing high performance liquid chromatography
DMSO	:	Dimethylsulfoxide
DNA	:	Deoxyribonucleic acid
dNTP	:	Dinucleotide triphosphate
dsDNA	:	Double strand DNA
dTTP	:	Deoxythymine triphosphate
EDTA	:	Ethylenediaminetetraacetic acid
ER	:	Endoplasmic reticulum
G	:	Guanine
G6PD	:	Glucose-6-phosphate dehydrogenase
G71R	:	Glycine to Arginine at codon 71
GS	:	Gilbert syndrome
Hb	:	Haemoglobin
Hi-Di	:	Highly deionized
HO	:	Haem oxygenase
HPLC	:	High-performance liquid chromatography

Kb	:	Kilo base pair
kg	:	Kilogram
LOH	:	Loss of Heterozygosity
m_1v_1	:	Mol volume
Mer	:	Merck
MGB	:	Minor groove binder
$MgCl_2$	:	Magnesium chloride
ml	:	Millilitre
mL/min	:	Millilitre per minute
mM	:	Micro mol
mm	:	Millimetre
mRNA	:	Messenger RNA
MSX1	:	Mash homobox1
n	:	Sample size
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NCBI	:	National Centre of Biotechnology Informatics
ng/µl	:	Nanogram/microliter
NICU	:	Neonatal intensive care unit
nm	:	Nanometre
NNJ	:	Neonatal jaundice
O_2	:	Oxygen
P229L	:	Proline to Leucine at codon 229
P229Q	:	Proline to Glutamine at codon 229
P364L	:	Proline to Leucine at codon 364
P364P	:	Proline to Proline at codon 364
PASW	:	Predictive analytic software

PCR	:	Polymerase chain reaction
RB1	:	Retinoblastoma protein
RBC	:	Red blood cell
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribonucleic acid
rpm	:	Round per minute
S258R	:	Serine to Arginine at codon 258
SB	:	Serum bilirubin
SD	:	Standard deviation
SNP	:	Single nucleotide polymorphism
SPSS	:	Science package social software
SSCP	:	Single Strand Conformation Polymorphism
T	:	Thymine
TBE	:	Tris base EDTA
TEAA	:	Triethylammonium acetate
T412S	:	Threonine to Serine at codon 412
U	:	Uracil
U/µl	:	Unit per micro litre
UDPGT	:	Uridine diphosphoglucuronyltransferase
UGT	:	Uridine glucuronyl transferases
UGT1A1	:	Uridine glucuronosyltransferase 1A1 isoform
USA	:	United State of America
USM	:	Universiti Sains Malaysia
UV	:	Ultraviolet
ρ	:	Piko
ρmol	:	Piko mol

LIST OF SYMBOLS

∞	: Infiniti
<	: Less than
>	: More than
$^{\circ}C$	: Degree Celsius
~	: Approximately
g	: Gram
α	: Alpha
β	: Beta
%	: Percentage
/	: Or
±	: Plus minus
P1	: Proportion of the G71R variant in non-jaundiced babies
P2	: Proportion of the G71R variant in jaundiced babies
Zα	: Value of the standard normal distribution cutting of probability α
Zβ	: Value of the standard normal distribution cutting of probability β

PENYARINGAN *UGT1A1* GEN DAN HUBUNGKAIT GENOTIP-PENOTIPIK DALAM PENYAKIT KUNING DARIPADA SAMPLE BAYI BARU LAHIR DI KELANTAN

ABSTRAK

Proses pengeluaran bilirubin adalah melalui proses glukuronidasi bilirubin yang terletak pada hati dan proses ini dimangkinkan oleh sejenis enzim iaitu Uridine glucuronosil transferase. Enzim ini dikodkan oleh gen iaitu gen *UGT1A1*. Dalam sesetengah populasi, mutasi pada gen ini akan menyebabkan berlakunya demam kuning. Walau bagaimanapun, data bagi populasi Melayu di Malaysia kurang diberi perhatian. Objektif kajan ini meliputi: mengetahui peratusan variasi pada ekson pada gen *UGT1A1* dalam populasi Melayu yang menghidap demam kuning atau tanpa demam kuning serta untuk menghubungkaitkan penemuan dari segi genotip dan data Penotipik. Kajian keratin rentas dilakukan di Kelantan, Malaysia. Kumpulan disahkan sebagai bayi dengan demam kuning (kumpulan demam kuning) dan kumpulan bayi tanpa demam kuning (kumpulan kawalan) terlibat dalam kajian ini. Darah diambil untuk ujian genetik. DNA diekstrak daripada darah sebelum kaedah tindak balas berantai polimerase (PCR) dilakukan. Kromatografi cecair denaturasi berprestasi tinggi (DHPLC) dilakukan bagi menyaring keseluruhan ekson di dalam *UGT1A1* gen dan subjek yang dikenal pasti mengandungi bentuk ganda dua pada graf DHPLC, akan melakukan proses penjujukan DNA bagi mengesahkan kehadiran mutasi atau SNP. Data klinikal di ambil sebagai sebahagian daripada kajian yang besar yang di jalankan. Data dimasukkan melalui SPSS dan dianalisis. 286 bayi terlibat di dalam setiap kumpulan, kumpulan demam kuning dan kumpulan kawalan. Sembilan variasi telah di temui dalam kajian ini. Variasi yang paling kerap ialah

G71R pada ekson 1, yang kerap ditemui pada populasi Asia yang lain. Hanya 4 daripada varisi itu pernah dilaporkan sebagai SNPs pada populasi yang lain. Manakala variasi yang lain, di laporkan sebagai mutasi yang baru. Walaupun banyak variasi dilaporkan pada kumpulan demam kuning berbanding kumpulan kawalan, namun tiada perbezaan secara statistik antara kedua-dua kumpulan secara statistiknya adalah saling tidak berkaitan. Kajian ini juga menunjukkan tiada kaitan antara kehadiran faktor penyakit kuning pada bayi dan hemolitik antara bayi kuning dengan dan tanpa variasi dalam *UGT1A1* gen. G71R telah dikenal pasti sebagai variasi yang utama dalam *UGT1A1* gen. Keputusan kajian ini masih belum menunujukan dengan jelas faktor menyebabkan berlakunya demam kuning.

SCREENING OF *UGT1A1* GENE AND THE GENOTYPE-PHENOTYPE CORRELATIONSHIP IN NEONATAL JAUNDICE FROM A SAMPLE OF NEWBORNS IN KELANTAN

ABSTRACT

The rate limiting step of bilirubin excretion is the glucuronidation of bilirubin in the liver, a process that is catalyzed by an enzyme, Uridine glucuronyl transferase. This enzyme is encoded by the *UGT1A1* gene. In several populations, mutations in this gene have been shown to cause neonatal jaundice. However data on the Malaysian Malay population are scanty at best. The objectives of this study included: to determine the frequency of variants in the exons of the *UGT1A1* gene in a population of term Malay neonates with jaundice and without jaundice, and to correlate the genotype finding with some phenotypic data. A cross sectional study was performed in Kelantan, Malaysia. A group of term jaundiced neonate (jaundice group) and a group of term non-jaundiced neonate (control group) were included in the study. Blood was taken for genetic testing. DNA was extracted from blood before polymerase chain reaction (PCR) was performed. Denaturing high performance liquid chromatography (DHPLC) was performed to screen the whole exons in the *UGT1A1* gene and for subjects that were identified to have a heteroduplex peak in DHPLC, sequencing was performed to confirm the mutation or SNP. Clinical data were collected as part of a larger study. Data were entered in SPSS and analyzed. Two hundred and eighteen six (286) neonates were included in each the jaundice and control groups. Nine variants have been identified in this study. The most common variant was the G71R variant in exon 1, which is common

in other Asian populations as well. Only 4 of the other variants detected in this study had been reported as SNPs in another population. Other variants appeared to be novel mutations. Even though variants were found in a higher number in the jaundice group than in the control group, the difference between the groups was statistically not significant. There were also no significant differences in severity of neonatal jaundice and in haemolysis between jaundiced neonates with and without identified variants in the *UGT1A1* gene. The G71R variant had been identified as the most common variant in the exons of the *UGT1A1* gene. The results of this study did not clearly show that this was a risk factor for neonatal jaundice.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Neonatal jaundice (NNJ) is the most common reason for hospital admission of new born neonates. In the American population, neonatal jaundice (NNJ) was reported to occur with a frequency of 60% in full term neonates and 80% in premature neonates (Agarwal *et al.,* 2007). An earlier study suggested that the incidence of neonatal jaundice was significantly lower among Americans from African ancestry compared to African populations that are living in Africa (Kaplan *et al.,* 2008). This suggests that environmental factors may play an important role in the pathogenesis of neonatal jaundice. On the other hand, East Asians were reported to have a much higher incidence of severe neonatal jaundice than Caucasians (Yamamoto *et al.,* 2002) and this difference persisted in a population of East Asians for several generations after migration to the USA (Miller, 2008). These data suggest that the increased incidence of NNJ in East Asians is due to genetic factors. So both environmental and genetic factors may play an important role in the pathogenesis of NNJ (Ardakani *et al.,* 2007) (Kaplan M *et al.,* 2005).

Hemolytic factors such as ABO incompatibility, Rhesus incompatibility, and glucose-6-phosphate dehydrogenase (G6PD) deficiency are among the best known causes of NNJ. In Malaysia all neonates are screened for G6PD deficiency with the hope of preventing kernicterus and bilirubin encephalopathy through early detection and treatment of jaundice. Furthermore neonates with NNJ requiring hospitalization are screened for the presence of ABO incompatibility and signs of hemolysis in the blood film. Other well known causes of neonatal jaundice that are routinely looked for in the neonates with NNJ requiring therapy, include polycythaemia, birth trauma, low birth weight, prematurity, poor breast feeding (Fabris *et al.*, 2009) and a positive family history (Rennie J *et al.,* 2010). Still a lot of neonates get severe NNJ and without any of the above risk factors being clearly identified and these neonates are labelled to have idiopathic NNJ or excessive physiological NNJ.

Several genetic risks may play an important role in this group with idiopathic NNJ. Mutations in a gene known as Uridine GlucuronosylTransferase 1A1 (*UGT1A1*) gene have been investigated as a genetic factor potentially contributing to NNJ. Mutations might appear either in the enhancer region, the promoter region, exons or introns. In the Caucasian population a particular mutation in the promoter region called A $(TA)_7TAA$ mutation is proven to be a risk factor for NNJ and results in a markedly reduced expression of the enzyme (Bosma *et al.*, 1995). In some East Asian populations (Japan and South Korea) a mutation in exon 1 of the *UGT1A1* gene has been identified as a risk factor for NNJ and was associated with a close to 60% expression of the enzyme (Yamamoto *et al.*, 1998).

In Malaysia, mutations in the *UGT1A1* gene promoter and enhancer regions have been investigated and were shown to be significantly associated with NNJ (Yusoff S

et al., 2010). There are few reports of small studies in the Malay population looking at mutations in exons (Sutomo *et al.*, 2004) (Yusoff *et al.*, 2006). Mutations in exons were also described by the same group of researchers in a Malay family resulting in a significant reduction of expression of the Uridine Glucuronosyltransferase (UGT) enzyme (Wada *et al.*, 2006).

The purpose of this study was to screen for mutations in the exons of the *UGT1A1* gene in a group of Malay neonates with neonatal jaundice and compare the incidence of such mutations between neonates with and without neonatal jaundice. Within the group of neonates with neonatal jaundice, an attempt was made to correlate the presence of exonic mutations with the severity of jaundice and signs of haemolysis.

In the remaining part of the introduction of this thesis, subsequently, the following topics will be covered. Neonatal jaundice will be defined after which briefly the bilirubin metabolism and some clinical aspects of neonatal jaundice will be covered. After that the author will focus in on the *UGT1A1* gene.

1.2 Definition of Neonatal jaundice (NNJ)

Neonatal jaundice is a yellow discoloration of the skin, mucosal membranes and white eye (sclera) in newborn neonates. It is related to an increase in serum bilirubin levels. Jaundice becomes apparent, only if the serum bilirubin levels increase to more than 90 μmol/L in the most cases. The jaundice usually starts in the face and progresses caudally as the serum bilirubin levels increase (Anthony and Stoll, 2007).

1.3 BILIRUBIN METABOLISM

Red blood cells (RBC) are produced in bone marrow and destroyed in the spleen. When the RBC are lysed, haemoglobin (Hb), that contains heme and globin is released. The globin part is broken down into amino acids and the iron is released from the heme group. The heme group is converted to biliverdin and later to bilirubin (Maisels and Watchko, 2000). Every 1 gram of Hb produces 35mg of bilirubin (bilirubin is produced at 8.5±2.3mg/kg/24 hours) (Watchko, 2000).

Bilirubin is a waste product of the heme metabolism. It is insoluble in water because of internal hydrogen bond interactions and it consists of four pyrrole and porphyrin rings (Fialova and Vejrazka, 2010). About 80% of the bilirubin molecules are derived from the catalysis of circulating haemoglobin (Hb). While another 20% is originated from other heme containing proteins, for example P450s and myoglobin (Tukey and Strassburg, 2000).

The bilirubin formed is unconjugated bilirubin (UB), which is lipid soluble. This molecule is transported in the plasma, bound to albumin to form bilirubin-albumin complex. When this bilirubin-albumin complex reaches to hepatocytes (liver cells), it will bind to the cell surface and bilirubin will be transported into the cell. In the liver, bilirubin will conjugate to glucuronic acid (making the molecule water soluble). This process is the rate limiting step in bilirubin excretion and is catalyzed by the uridine diphosphoglucuronyltransferase (UDPGT) enzyme. Only after conjugation it can be excreted into the bile to the small intestine. Some of conjugated bilirubin (CB) in the large intestine is metabolized to stercobilinogen before it is oxidized and changed to stercobilin which gives the brown colour to the faeces. Another part of the conjugated bilirubin is converted to urobilinogen which

is reabsorbed and excreted in the urine in an oxidized form, called urobilin. A fraction of bilirubin from the faeces will be reabsorbed into blood via portal circulation (enterohepatic circulation). During the foetal life in the mother's womb, bilirubin is eliminated through the placenta via the umbilical cord, while during neonates, the liver will eliminate it in the conjugated form of bilirubin before it is moved to the gastrointestinal tract (Hansen, 2000).

If the unconjugated bilirubin (UB) does not excreted fast enough by the liver, the serum bilirubin will increase and bilirubin is deposited in tissues in the body leading to NNJ. This UB has the ability to cross the blood brain barrier (BBB) and cause kernicterus or bilirubin encephalopathy. Kernicterus appears when the level of serum bilirubin becomes too high and it can be confirmed during autopsy. It will give a yellow colour to the hippocampus, basal ganglia, nuclei of cerebellum and brain stem (Bhutani and L., 2005).

In most of the cases, NNJ occurs due to unconjugated bilirubin formation that is caused by immaturity of hepatocytes leading to low activity of the UDPGT. Another factor of NNJ is the short life span of RBC (between 45-90 days) in neonates as compared to adults (about 120 days) that lead to overproduction of serum bilirubin in the neonates (Moerschel *et al.*, 2008). Figure 1.2 and 1.3 shows the normal and abnormal bilirubin pathways respectively.

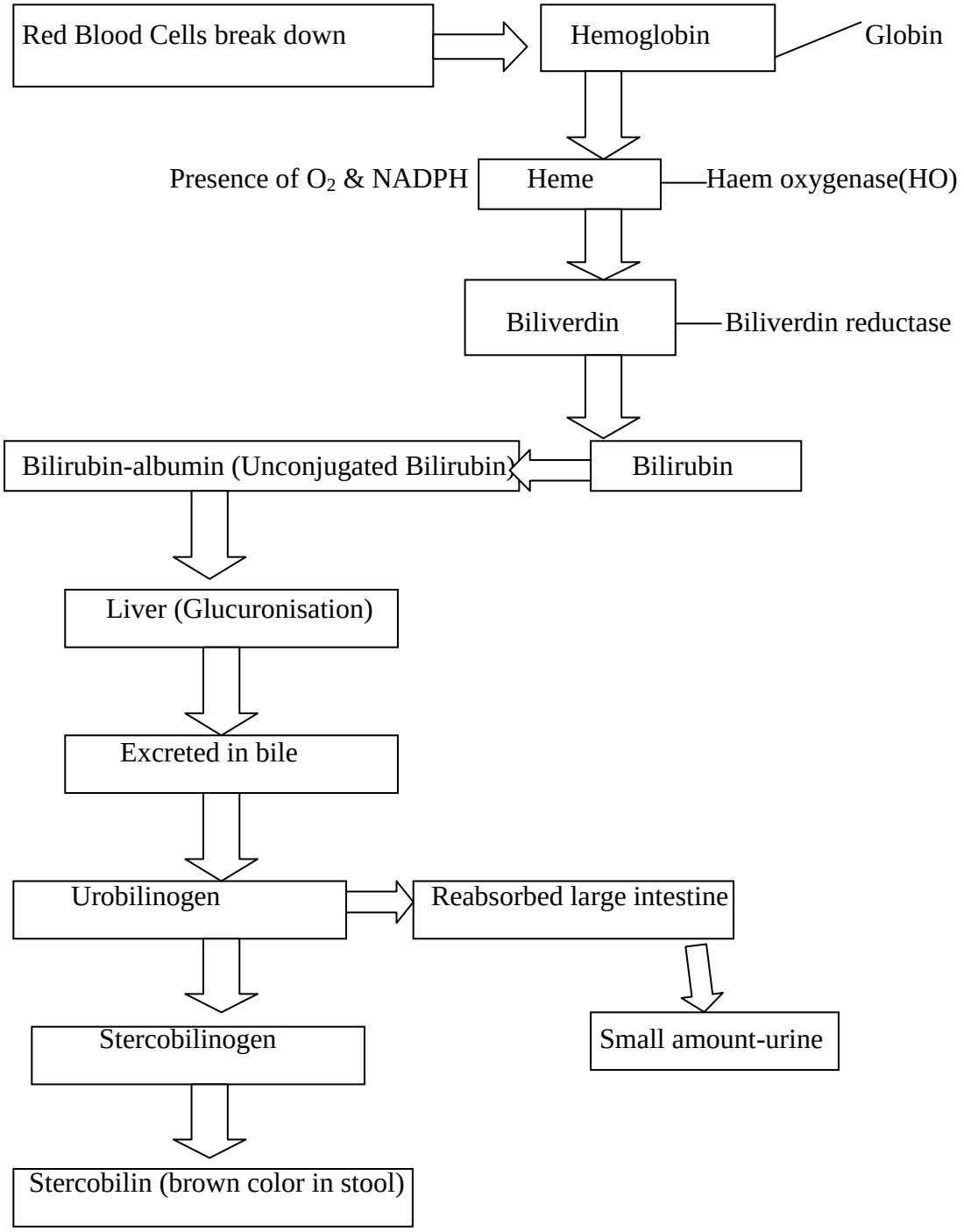

Figure 1.1: Normal Pathway of bilirubin metabolism

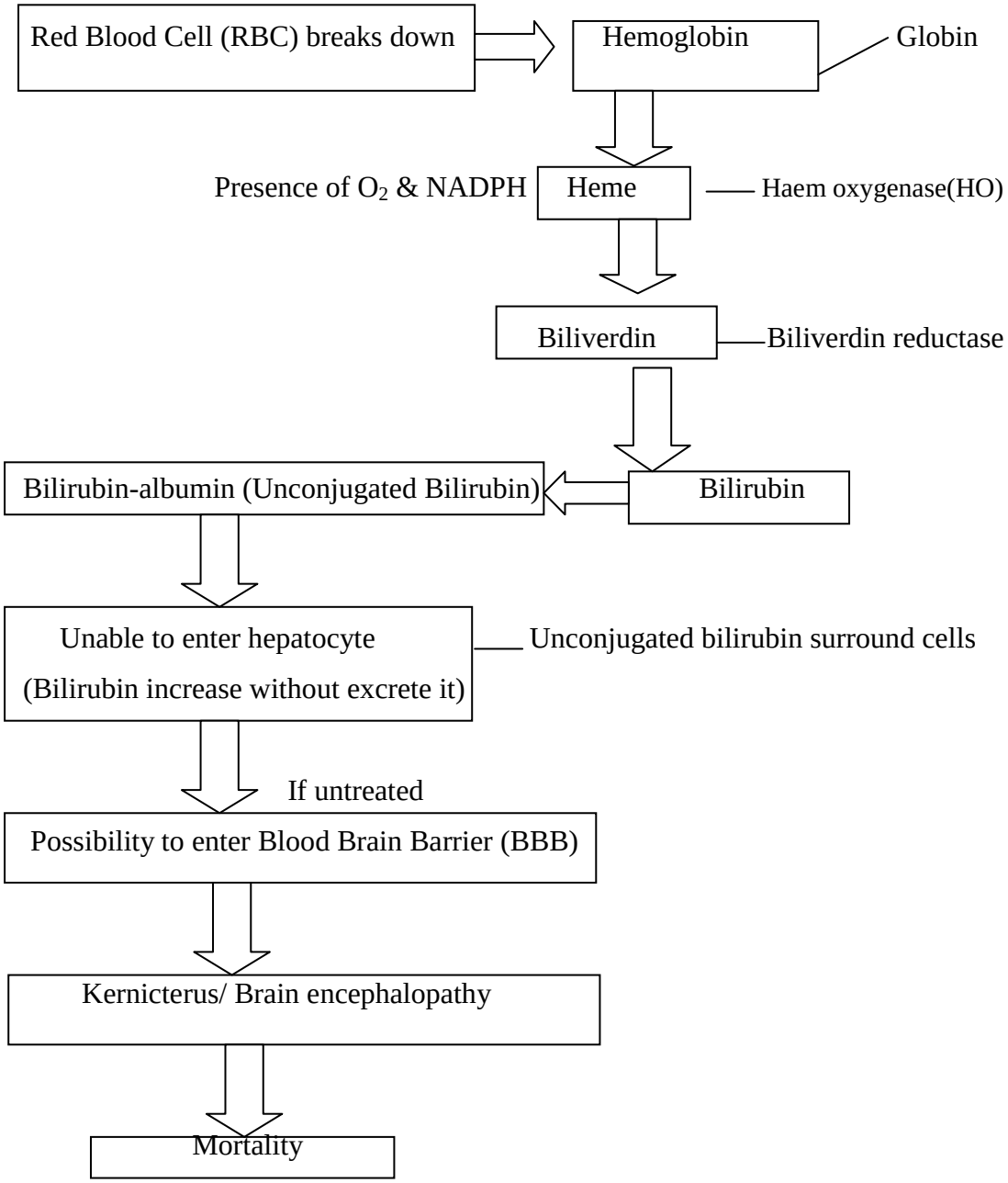

Figure 1.2: Abnormal Pathway in bilirubin metabolism

1.4 Clinical aspects of neonatal jaundice (NNJ)

1.4.1 Unconjugated bilirubin (Indirect hyperbilirubinemia)

Unconjugated bilirubin consists of lipid soluble or fat soluble molecules. Indirect hyperbilirubinemia occurs when the serum bilirubin level exceeds 34.2µmol/L. Indirect hyperbilirubinemia leads to accumulation of free bilirubin in certain parts of the body and most importantly it can cross the blood brain barrier (BBB) and accumulate in the brain leading to kernicterus and neurological disease. It is the small fraction of unconjugated bilirubin in the plasma that is not bound to albumin (unbound bilirubin) that can move into the brain. Kernicterus was first described in 1847 by Hervieux (Juretschke, 2005) and it was observed from tissues of the brain during an autopsy where the yellow staining had appeared. Presence of endogenous or exogenous binding competitors to albumin such as certain drugs can decrease binding affinity of albumin to bilirubin and increase the risk of kernicterus.

1.4.2 Conjugated bilirubin (Direct hyperbilirubinemia)

Conjugated bilirubin is identified as a water soluble molecule that moves through the bile duct to the intestine (Odell, 1980). The conjugation process involves the binding of 2 molecules of glucuronic acid to bilirubin, a process catalysed by the uridine glucuronosyl transferase (UGT). Direct hyperbilirubinaemia in the neonate is generally due to hepatocellular diseases and infectious or metabolic disorders (Klein *et al.*, 2010).

1.5 Common causes of neonatal jaundice

1.5.1 Physiological jaundice

Physiological jaundice is a mild form of jaundice that occurs in almost 60% of term newborns and in about 70% of preterm neonates. It appears after 24 hours (within 48 to 72 hours) of life (Saxena, 2008). The peak serum bilirubin levels are reached at the 3rd or 4th day of life for full term neonates and it disappears after 14 days of life. Usually, it is caused by a combination of increased bilirubin production due to a shorter lifespan of RBC containing foetal haemoglobin and immaturity of the liver leading to accumulation of unconjugated bilirubin. Physiological jaundice consists of two phases. In phase I serum bilirubin will rapidly increase in the first three days, while in phase II, it will decreases slowly and reaches the normal range by day 11-14 of life (Madan *et al.*, 2005). Physiological jaundice is less severe than pathological jaundice.

1.5.2 Pathological jaundice

Another type of jaundice is pathological jaundice. This type of jaundice commonly appears in the first 24 hours of life (Jack, 2011) and requires treatment. It can be seen when serum bilirubin levels exceed the 95th percentile as defined in a nomogram (Kaplan *et al.*, 2006).

Pathological jaundice can be caused by haemolytic and non-haemolytic (Kaplan *et al.*, 2006) factors.

1.5.2.1 Haemolytic factors

Factors associated with an increase in red blood cell (RBC) breakdown are many and include glucose-6-phosphate dehydrogenase (G6PD) deficiency, ABO incompatibility, Rhesus incompatibility, other blood group incompatibilities, polycythaemia, birth trauma, sickle cell anaemia, and other abnormalities of the RBC. In the next two subchapters a brief elaboration will be given on G6PD deficiency and ABO incompatibility.

1.5.2.1 (i) Glucose-6-Phosphate Dehydrogenase (G6PD) Deficiency

Glucose-6-phosphate dehydrogenase or G6PD is known as an RBC enzyme deficiency where the decrease of enzyme in the body might lead to haemolysis. The incidence of G6PD deficiency is higher in people from Africa, the Middle East, Arabian Peninsular, Mediterranean area and Southeast Asia including Thailand and Malaysia (Cappellini and Fiorelli, 2008). Since the 1980s, screening of G6PD in neonates has been practiced in Malaysia for early detection and treatment of neonatal jaundice and affected neonates will be observed in the ward (Ainoon *et al.*, 2003).

The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (Cappellini and Fiorelli, 2008) is used as a co-enzyme for G6PD which is useful to maintain the level of glutathione that will protect the RBC from oxidative stresses.

One of the well known complications of G6PD deficiency is NNJ which usually starts on day 2 to 3 of life and can be very severe (Bhutani *et al.*, 2004). In Malaysia, a study suggested a high incidence of G6PD deficiency in the Malay and Chinese populations (Singh, 1986). However it is lower in the Indian population. Its

incidence in males is much higher than in females because G6PD deficiency is an X-linked recessive disorder (Garg *et al.*, 1984).

1.5.2.1 (ii) ABO Incompatibility

ABO incompatibility cases commonly appear when a mother with blood group 'O' is having or carrying a child with blood groups either 'A' or 'B' (Mourant, 1977). In neonates with ABO incompatibility, NNJ usually appears during the first 24 to 48 hours of life (Bhutani *et al.*, 2004) . However, ABO incompatibility not only occurs in neonates of mothers with 'O' blood type. It also can happen if mothers with blood group A have neonates carrying blood group B and if mothers with blood group B have neonates carrying blood group A (Iannelli, 2006).

A previous study by Kalakheti et al reported that neonates with ABO incompatibility had a two times higher chance to get hyperbilirubinaemia compared to neonates with other haemolytic risk factors (Kalakheti *et al.*, 2009).

1.5.2.2 Non-hemolytic factors

In few cases, neonatal jaundice may appear due to non-hemolytic factors. These conditions include:

1. Inborn errors of metabolism such as Crigler- Najjar Syndrome types I, II and Gilbert Syndrome (Huang *et al.*, 2001). These are described in more details below.

2. Impaired bilirubin excretion leading to neonatal cholestasis, for example in Dubin-Johnson syndrome or infections such as urinary tract infection (UTI) and sepsis (MacDonald *et al.*, 2005).

3. There are two types of NNJ related to breast feeding. One is breast feeding jaundice, which is related to inadequate milk intakes early in life and this is associated with early onset jaundice. A second type is breast milk jaundice, related to a substance present in breast milk (3-α-20β pregnanediol) that may cause NNJ in 1 to 2% of breastfed neonates. NNJ symptoms usually start on day 4 to day 7 and can last 3 to 10 weeks, or even longer. Both types are associated with an increase in enterohepatic circulation (Schmitt, 2009).

Associated factors	Effect on neonatal serum bilirubin levels		
	Increase	Decrease	No effect
Ethnicity	East Asian Native American Greek	African American	-
Genetic or familial	Previous sibling with jaundice Variant promoter or mutation of *UGT1A1* gene associated with Gilbert's syndrome	-	-
Maternal	Older mothers Diabetes Hypertension Oral contraceptive use at time of conception First-trimester bleeding Decreased plasma zinc level	Smoking	-
Drugs administered to mother	Oxytocin Diazepam Epidural anesthesia Promethazine	Phenobarbital Meperidine Reserpine Aspirin Chloral hydrate Heroin Phenytoin Antipyrine Alcohol	Beta-adrenergic agents
Labor and delivery	Premature rupture of membranes Forceps delivery Vacuum extraction Breech delivery	-	Fetal distress

Infant	Low birth weight	-	-
	Low gestation		
	Male gender		
	Delayed cord clamping		
	Elevated cord blood bilirubin level		
	Delayed meconium passage		
	Breast feeding		
	Caloric deprivation		
	Larger weight loss after birth		
	Low serum zinc and magnesium		
Drugs administered to infant	Chloral hydrate	-	-
	Pancuronium		
Other	Altitude	-	-
	Short hospital stay afterbirth		

Table 1.1 The factors associated with neonatal jaundice (Adapted from (Maisels and Watchko, 2000).

1.6 Clinical syndromes associated with mutations in the *UGT1A1* gene

Gilbert syndrome (GS) and Crigler-Najjar syndrome (type I and type II) are the clinical syndromes which are commonly related to mutations in the *UGT1A1* gene associated with NNJ. Many studies reported that a decreased activity of the uridine diphosphoglucuronosyltransferase enzyme (UDPGT) in the liver, resulting in increases of unconjugated bilirubin levels is due to mutations in the *UGT1A1* gene (Bosma *et al.*, 1995) (Strassburg and Manns, 2000). These clinical syndromes are classified into three forms based on serum bilirubin levels (Huang *et al.*, 2001).

- The most severe form is Crigler-Najjar syndrome type I (CN I).

- Intermediate severity is seen in Crigler-Najjar syndrome type II (CN II).

- The mildest form is Gilbert syndrome.

In the Malay population reports about these clinical syndromes are scanty but mutations typically associated with Gilbert syndrome have been reported (Yusoff *et al.*, 2006).

1.6.1 Gilbert syndrome (GS)

GS was discovered in 1901 by Gilbert and Lereboullet, and was described as ``La cholemie simple familiale" (Sampietro and Iolascon, 1999). GS is caused by a reduction in glucuronidation activity of the *UGT1A1* enzyme in the liver by 25-50% from the normal range (Innocenti *et al.*, 2002). Mutations in the *UGT1A1* gene that may cause GS were only discovered in 1995 (Bosma *et al.*, 1995).

GS is a benign condition with a mild form of often fluctuating hyperbilirubinaemia. It might involve a single or complex locus that encodes for the enzyme, bilirubin-UDP-glucuronosyltransferase (Sampietro and Iolascon, 1999).

In Malaysia, mutations in *UGT1A1* that lead to GS were first reported in 2004 and they were found to be quite common (around 6.3%) in the studied Malay population (Sutomo R *et al.*, 2004).

Many studies showed that mutations of the *UGT1A1* gene related to GS were commonly observed in the promoter region with addition of 2 base pairs (bp) of Thymine (T) and Adenine (A) nucleotide in an area known as TATA box (Iolascon A et al., 1999) (Beautler E et al., 1998). The normal repetition of TA is 6 and an addition of TA as seen in the A $(TA)_7$TAA mutation, also known as *UGT1A1**28, might lead to Gilbert syndrome. The addition of TA causes impairment of proper message transcription that can cause a decrease of *UGT1A1* activity in the liver (Bosma *et al.*, 1995).

A study from Scotland also suggested that GS not only contributed to prolonged jaundice in breast fed infants but also led to pharmacological variations in drug glucuronidation and sometimes unexpected toxicity from therapeutic agents (Burchell and Hume, 1999).

In Liverpool, GS had been studied as the most inherited metabolic liver disorder (around 6%) in a Caucasian population (Owens and Evans, 1975). This report had been supported that the A $(TA)_7$ TAA mutation in the promoter region is the most common cause of GS in Caucasians (Beutler *et al.*, 1998) compare to Asian countries. In Asia, mainly in East Asians countries, it has been proven to be more

commonly due to a mutation in exon 1 known as the G71R mutation (Maruo *et al.*, 1999b).

1.6.2 Crigler-Najjar Syndrome (CN)

Crigler-Najjar syndrome (CN) was discovered in 1952 by 2 researchers, Crigler and Najjar. CN is also known as congenital non-obstructive non-hemolytic hyperbilirubinemia (John F.Crigler, 1952)

Mutations in the *UGT1A1* gene leading to unconjugated bilirubin in CN was first reported by Ritter et al in 1992 (Ritter *et al.*, 1992). CN had been classified into two types known as Crigler-Najjar Syndrome type I (CN I), discovered by Crigler and Najjar and Crigler-Najjar Syndrome type II (CN II) discovered by Arias, based on the level of serum bilirubin and the treatments (Arias, 1962).

CN is marked by partial or total deficiency of the glucuronyl-transferase enzyme and might cause fatal hyperbilirubinaemia in neonates if untreated. In severe cases, this syndrome may lead to brain damage (kernicterus) to neonates and early screening needs to be performed to avoid it. CN can be caused by mutations in intron (non-coding) or exon (coding) regions of the *UGT1A1* gene.

1.6.2 (i) Crigler-Najjar Syndrome Type I (CN I)

Crigler Najjar syndrome type I or CN I is inherited in an autosomal recessive way and is a rare syndrome (Arias *et al.*, 1969). Studies suggested that CN I is caused by a complete absence of UDPGT enzyme activity toward bilirubin in the hepatocytes.

CN I occurs in 1:1,000,000 newborns and can be observed in the first day of life with high levels of serum bilirubin (Labrune, 2004). Neonates with CN I do not

respond to phenobarbital treatment. CN I can be caused by several types of mutation such as homozygous nonsense, frameshift or homozygous missense mutation (Kadakol *et al.*, 2000). CN I is mainly associated with the mutations in exons 2-5 that might affect the termination codon (Clarke *et al.*, 1997) and premature truncation of the *UGT1A1* gene (Kadakol *et al.*, 2001). Mutation in CN I was first reported in 1992 (Ritter *et al.*, 1992) and CN I can be diagnosed using high-performance liquid chromatography (HPLC) for analysis of the bile or tissue enzyme assay from liver biopsy. A study in 2007 showed that CN I also often appeared in the children from consanguineous parents as described in Slovakia's population (Zmetakova *et al.*, 2007).

1.6.2 (ii) Crigler-Najjar Syndrome Type II (CN II)

Crigler- Najjar syndrome type II or CN II is also mainly inherited as an autosomal recessive trait (Arias *et al.*, 1969) even though reports of autosomal dominant inheritance are available (Arias, 1962). The serum bilirubin level is markedly elevated but less than in CN I (Jansen, 1999).

Enzyme activity is severely reduced in CN II but it can be induced following phenobarbital administration. CN II rarely causes damage to the central nervous system (CNS) and the majority of the patients can survive until adulthood without any complications. CN II has been more frequently reported in Asian populations as compared to other populations (Yamamoto K *et al.*, 1998).

1.7 Uridine Diphosphoglucuronate Glucuronosyl Transferase (UDPGT) 1A gene

UDPGT is an enzyme found in many cells in the human body such as the liver, intestines, kidneys, lungs and adrenals. Uridine glucuronyl transferases (UGT) consist of a large superfamily and are classified into two major isoforms based on the nucleotide sequence for the identification of their own isoforms, UGT1 and UGT2. These isoforms provide glucuronidation of bilirubin, oestriol, oestradiol, quinols and phenols. It represents a super family of microsomal membrane-bound enzymes that catalyze conjugation that is not only limited to bilirubin (Kaplan and Hammerman, 2005).

UGT1 encodes a biochemical reaction for the conjugation of glucuronic acid. This isoform also mediates glucuronidation of other aglycone substrates in *UGT1A* locus. *UGT1A1* is the only isoform that contributes substantially to the bilirubin glucuronidation transferase (Kaplan and Hammerman, 2005).

The length of the gene encoding the UGT1A family is 218kb, consisting of 13 variable isoforms in exon 1 and all these isoforms are spliced into 4 common exons (exon 2 to exon 5). From the 13 variable isoforms, only 9 isoforms encode for protein (1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, and 1A10) while another 4 isoforms are pseudogenes, not functionally important in the UGT1A family (1A2, 1A11, 1A12 and 1A13). Only variable exon A1 is important in bilirubin conjugation and the others play a role in the detoxification of a diverse range of chemical substances (Kaplan and Hammerman, 2005). Exons 2 to 5 are responsible in specific signal and transcription processing splices of mRNA from single variable exons to the common exons.

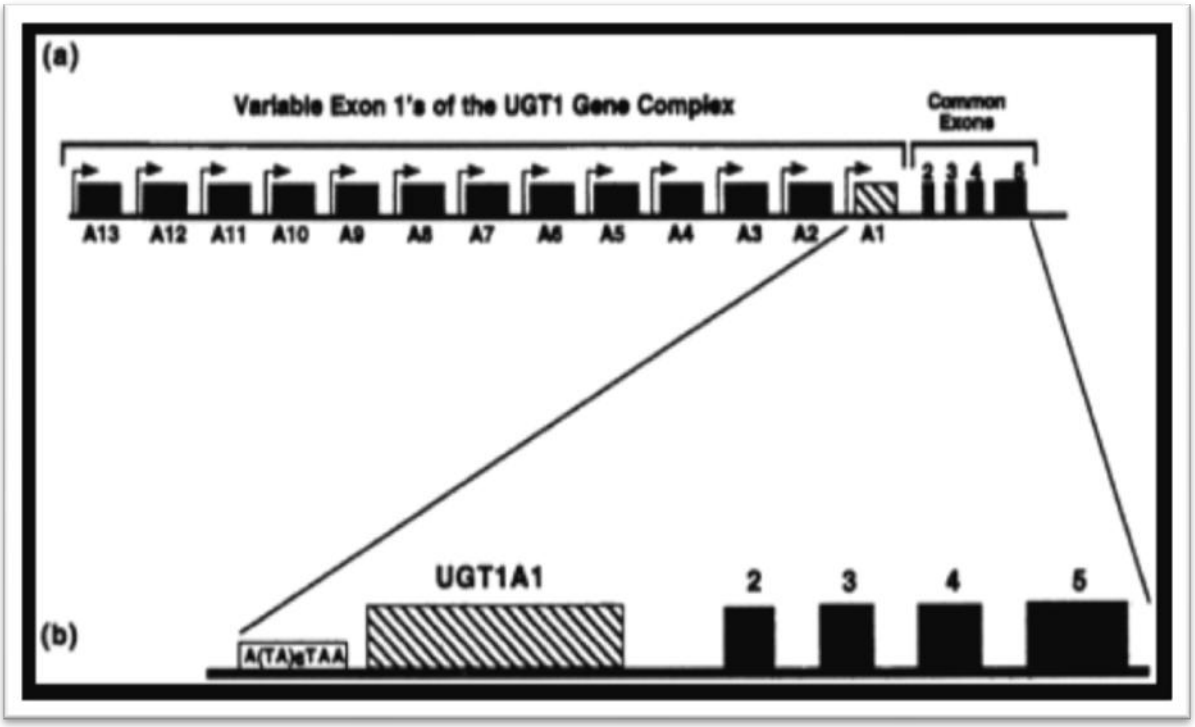

Figure 1.3: Human UGT1 gene locus.

(a) Schematic representation of the genomic structure of the UGT gene complex.

(b) Exploded view of exon 1A1 and common exons 2-5 of the gene complex that have been identified as sites for genetic mutations associated with absent or decreased of UGT activity that cause deficiencies of bilirubin conjugation. Used with permission from Clarke (Clarke *et al.*, 1997)

1.8 Bilirubin Uridine Diphosphate-Glucuronosyltransferase 1A1 (*UGT1A1)* gene

The bilirubin uridine diphosphate-glucuronosyltransferase 1A isoform 1 or *UGT1A1* gene is located in the q arm of chromosome 2, 2q37.1 (see figure 1.4). Other names for the *UGT1A1* gene include GNT1, Uridine Diphosphate Glucuronosyltransferase (UDPGT) and HUG-BR1. It consists of 5 exons (1-5, where exon 1 is spliced to exon 2-5) and the specific promoter region. This gene is located at the 3' end of the *UGT1A* locus (Costa *et al.*, 2006).

The common exons in UGT1A family (exons 2-5) are located at 3'end which encode carboxylterminal domain for all *UGT* isoforms and bind with UDP-glucuronic acids. Each isoform in the UGT1A family has the unique exon in exon 1 and also their own promoter which is located in the 5' N-terminal domain. The *UGT1A1* gene has an approximate length of about 13 kb (13,027bp) which is not including other genes that overlap in the UGT1A family transcripts. Bilirubin uridine diphosphate-glucuronosyltransferase (B-UGT) is the key enzyme for bilirubin metabolism, and decreasing of *UGT1A1* gene activity leads to unconjugated hyperbilirubinemia that may result in Gilbert Syndrome or Crigler-Najjar Syndrome. Mutations that appear in exons and promoter of the *UGT1A1* gene may cause structural or functional abnormalities of the enzyme, causing impaired bilirubin conjugation, leading to hyperbilirubinemia (Kaplan *et al.*, 2003).

Mutations of the *UGT1A1* gene may affect the bilirubin glucuronidation activity that is leading to these inherited disorders. Mutation in the promoter region reduces the *UGT1A1* gene expression while mutation in the coding region cause dysfunction of the *UGT1A1* gene and also an abnormality of the gene structure (Sun *et al.*, 2007).

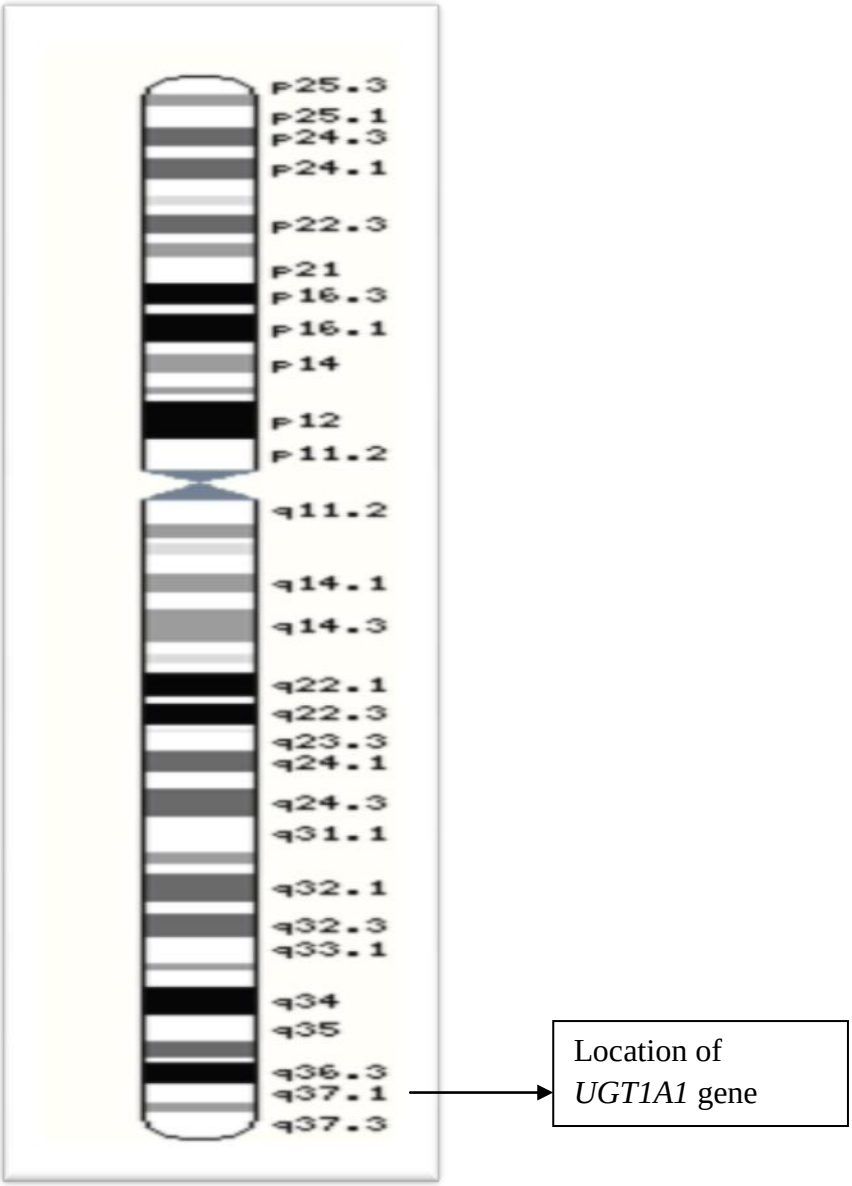

Figure 1.4 Location of *UGT1A1* gene in chromosome 2 (2q37.1)

Adapted from:

http://www.ensembl.org/Homo_sapiens/Location/Chromosome?g=ENSG00000241635;r=2:234669368-234682419;t=ENST00000305208

1.9 Mutations in the *UGT1A1* gene

Mutations of the *UGT1A1* gene can be classified into three groups:

1. Mutations that result in reduced production of the enzyme (Guillemette C., 2003).
2. Mutations that give rise to the synthesis of an enzyme that is structurally and/or functionally abnormal (Sampietro M *et al.*, 2003).
3. Mutations which completely abolish the expression of the affected allele (Sampietro M *et al.*, 1999).

Mutation is defined as a permanent change in the genome, either in DNA or RNA sequence which cause changes in amino acids or proteins related to that particular gene. Mutations may occur due to radiation, viruses and mutagenic chemicals which cause errors during meiosis or DNA replication (Bertram, 2001).

Mutations in the *UGT1A1* gene occur in the coding region, known as exon and in the non coding region, known as intron. Since in the middle of 1980s more prevalence of mutations in the *UGT1A1* gene had been discovered and mutation that is responsible for Gilbert syndrome was first described in 1995 by Aono et al (Aono *et al.*, 1995).

As discussed above (section on Gilbert Syndrome), an important mutation in Caucasians is located in the promoter region. Association of mutations in the promoter region and enhancer region have been found to be significant risk factors for NNJ (Bosma *et al.*, 1995) (Yamamoto *et al.*, 2002) and recently this has also

been confirmed in a Malay population (Yusoff S, 2010). The focus of the current study is however on the exonic region of the *UGT1A1* gene.

One of the exonic mutations that seems to play a real important role in neonatal jaundice in Asians (Long *et al.*, 2011) is the G71R mutation. It is a common mutation of the *UGT1A1* gene and has been widely reported in East Asian countries such as Taiwan, China and Japan.

In Southeast Asian populations, this mutation of the *UGT1A1* gene was believed to be first described in a Thai population (Sutomo *et al.*, 2002). Other reports about Southeast Asia include studies done in Singapore (Zhou *et al.*, 2009), Indonesia (Sutomo *et al.*, 2004) and Malaysia (Yusoff *et al.*, 2006) (Boo *et al.*, 2009). The findings in each of these studies are compared to the findings in the current study in the discussion part of this thesis. The G71R mutation is located in exon 1 of the *UGT1A1* gene and comprises of a substitution of Guanine to Adenine (G to A) at nucleotide 211. It causes a change of amino acids from glycine to arginine at codon 71 (G71R). The G71R mutation seems to be rare in Caucasian populations (Narter *et al.*, 2011) and because of the high prevalence of this mutation, in Asian population, it had been considered as polymorphism for Asian people (Takeuchi *et al.*, 2004).

A study in Japan showed that the G71R mutation, either in heterozygous or homozygous trait, may decrease the enzyme activity by 32.2% and 60.2% respectively using in- vitro expression (Yamamoto *et al.*, 1998). This condition may

lead from mild to moderate delay of bilirubin elimination in human (Sun *et al.*, 2007).

Another major mutation in Asian populations, mainly in the Japanese population, related to NNJ is the P229Q or Pro229Gln mutation which is also located at exon 1 of the *UGT1A1* gene. This mutation causes substitution of nucleotide Cytosine (C) to Adenine (A) at nucleotide 686 (Saeki *et al.*, 2003). There are studies suggesting that a combination of mutations (TATA box with G71R or P229Q) increases the serum bilirubin levels in neonates (Kamisako, 2004).
However, a study in Taiwan suggested that the combination of promoter or G71R and P229Q might not affect the serum bilirubin level significantly in neonates (Hsieh *et al.*, 2001).

As mutation at the same codon has also been observed in African-American population but a different nucleotide was involved. It caused a change of nucleotide from Cytosine (C) to Thymine (T) and is known as P229L, causing an amino acid change from Proline to Leucine (Kaniwa *et al.*, 2005b).

Besides G71R and P229Q mutations in exon 1, the Y486D in exon 5 of the *UGT1A1* gene had been suggested to contribute in the development of neonatal jaundice (Takeuchi *et al.*, 2004). The Y486D mutation causes a substitution of nucleotides (from Thymine (T) to Guanine (G) at position number 1456, leading to an amino acid change, Tyr486Asp. This results in a decrease of 7% of the *UGT1A1* gene enzyme function (Wu *et al.*, 2008).