INTRODUCTION
1. INTRODUCTION

Neonatal jaundice is caused by an increased production of bilirubin from senescent fetal red blood cells and/or limited bilirubin elimination in the newborn infant. Although neonatal jaundice is a natural and transitional phenomenon, some newborn infants develop severe hyperbilirubinemia. In these cases, unconjugated bilirubin in the serum may cross the blood-brain barrier and cause bilirubin encephalopathy (acute bilirubin intoxication in the brain) or kernicterus (chronic bilirubin intoxication in the brain). Here, “kernicterus” is used to indicate the chronic symptoms of bilirubin intoxication in the brain, although kernicterus has also been used as a histopathological term, meaning yellow discoloration of the nuclei. Bilirubin encephalopathy results in acute manifestations of bilirubin toxicity in the first weeks after birth such as lethargy, poor feeding, hypertonia, irritability and seizure. Kernicterus results in chronic and permanent clinical sequelae of bilirubin toxicity such as choreoathetoid cerebral palsy, central neural hearing loss, palsy of the vertical gaze and tooth enamel hypoplasia (American Academy of Pediatrics Subcommittee on Hyperbilirubinemia, 2004).

Racial variations in the incidence of neonatal hyperbilirubinemia have been recorded. The peak serum levels of unconjugated bilirubin in full-term Asians and American-Indian neonates are double than those in white and black populations (Halamek et al., 1997). The incidence of severe hyperbilirubinemia and kernicterus is also higher among Asian newborns (Maisel, 1994). Khoury et al., found that the risk of severe jaundice in newborns with one or more prior siblings who were jaundiced in infancy was 12.5 times higher than the risk in infants whose prior siblings were not jaundiced, even when obvious icterogenic factors were corrected (Khoury et al., 1998). These findings suggest that genetic factors may be involved in the development of severe neonatal hyperbilirubinemia.

It is of concern that early discharged infants may develop extremely high bilirubin levels at home. Therefore, it is a prerequisite to identify the infants at risk for developing severe hyperbilirubinemia before discharge (Maisels, 2009).

Some genetic diseases are associated with pathological conditions that may cause or aggravate neonatal jaundice. Thus, the knowledge of these diseases may be helpful in
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identifying the infants at risk. In this study, we examined the genetic disorders that may be a risk factor for neonatal jaundice.

Bilirubin is mainly produced in its unconjugated form by the turnover of erythrocytes. It is transported into the liver cell by the organic anion transporter 2 (OATP 2) (Cui et al., 2001), and it is then conjugated with glucuronic acid through reaction with UDP-glucuronosyltransferase 1A1 (UGT1A1) in the liver before being excreted into the bile (Clarke et al., 1997). The bilirubin level in neonates is much higher than in adults because the life span of the erythrocytes is relatively short and the capacity for bilirubin elimination is lower than in adults (Ives, 1999; Halamek et al., 1997). The incidence of severe hyperbilirubinemia and kernicterus is also higher among newborn Asian infants (Ives, 1999). These findings suggest that genetic factors may be involved in the development of severe neonatal hyperbilirubinemia. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common genetic defect, affecting 400 million individuals worldwide (Beutler, 1994). Furthermore, G6PD deficiency was the main risk factor for development of severe hyperbilirubinemia in Taiwanese neonates in the past (Yu et al., 1992). The situation has improved, however, since neonatal G6PD screening tests and health education were instituted at the Cathay General Hospital (CGH) in Taipei and nationwide in Taiwan (Yu et al., 1992; Huang et al., 1996).

Uridine diphosphoglucuronate glucuronosyl transferase 1A1 (UGT1A1) deficiency is a hereditary abnormality in the activity of the critical enzyme in the bilirubin glucuronidation pathway (Bosma et al., 1994). Bilirubin is derived from the breakdown of haemoglobin due to senescence of red blood cells. After transportation into hepatocytes, bilirubin is conjugated with glucuronic acid in the presence of UGT1A1. The conjugated bilirubin is hydrophilic, which makes it easier to be excreted into the bile (Gollan et al., 1996). There are two UGT1A1 deficiency syndromes, Gilbert’s syndrome (GS) and Crigler-Najjar syndrome (CN). GS (mild phenotype) and CN Type 2 (CN2; intermediate phenotype) result from a partial deficiency of UGT1A1, while CN Type 1 (CN1; severe phenotype) results from a complete deficiency of UGT1A1. Among UGT1A1 deficiencies, GS is very common, affecting about 6% of the population (Owens and Evans, 1975). In contrast, CN is very rare, with a frequency of 0.6 per million (van der Veere et al., 1996). CN1 patients develop severe hyperbilirubinemia in the first 2 to 3 days after birth, and often require exchange transfusions.
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Brain damage can occur at any time in CN1 patients, even in adulthood. CN2 and GS patients usually show less severe hyperbilirubinemia, although some infants show severe jaundice in neonatal period and needed some treatments such as phenobarbital administration or phototherapy (Gollan et al., 1996; Ciotti et al., 1998).

The UGT1A1 gene belongs to the UGT1A gene complex on chromosome 2 (van Es et al., 1993). Three major polymorphic mutations, variant TATA box, c.211G>A and c.-3279T>G, have been found in different populations with different prevalence (Innocenti et al., 2005). Variant TATA box with an additional TA insertion, A(TA)7TAA, located in the proximal promoter region of UGT1A1, was first found in patients with CN2 and GS (van Es et al., 1993). Subsequently, the variant TATA box was also found in CN1 (Ciotti et al., 1998). The A(TA)7TAA mutation does indeed reduce the UGT1A1 transcriptional activity (Bosma et al., 1995). Bancroft et al. showed that the A(TA)7TAA mutation accelerates development of neonatal jaundice (Bancroft et al., 1998). In the Caucasian population, the frequency of homozygosity for A(TA)7TAA in infants with neonatal jaundice was significantly higher than in normal infants (Laforgia et al., 2002). However, the A(TA)7TAA mutation may not be sufficient for the development of complete GS: GS patients with A(TA)7TAA may carry other additional mutations in UGT1A1. Some studies have reported that most GS patients with homozygosity of A(TA)7TAA were also homozygous for another mutation, c.-3279T>G (Maruo et al., 2004; Costa et al., 2006; Ferraris et al., 2006).

The c. 211G>A mutation is the most common mutation in the East Asian population (Akaba et al., 1998). This mutation replaces a glycine at codon 71 with arginine (G71R), leading to a decrease in UGT1A1 enzyme activity. The c.211G>A mutation was first found in a Japanese male with CN2 (Aono et al., 1993). In the Japanese population, the allele frequency of c.211G>A in infants with neonatal jaundice was significantly higher than in control infants (Maruo et al., 1999).

A variation in the promoter area or within the coding region of the UGT1A1 gene was associated with neonatal hyperbilirubinemia in whites and in Japanese and Taiwanese, respectively (Bancraft et al., 1998; Akaba et al., 1998; Maruo Y et al., 1999; Huang et al., 2002). It has been determined that the A(TA)7TAA promoter variant or homozygous G to A variation at nucleotide 211 in the UGT1A1 gene is an additive risk factor for neonatal hyperbilirubinemia in G6PD-deficient white (Kaplan et al., 1997; Galanello et
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al., 1999; Iolascon et al., 1999) and Taiwanese neonates (Huang et al., 2002). The occurrence of the A(TA)7TAA allele was relatively rare (14.3 versus 40%) and that the variation rate within the coding region of the UGT1A1 gene was much higher (29.3 versus 0.1%) in Taiwanese compared with whites (Huang CS et al., 2000). Previous research indicated that the variation at nucleotide 211 of the UGT1A1 gene was highly related to neonatal hyperbilirubinemia (Huang et al., 2002; Huang et al., 2002).

These days, there have been many studies on the UGT1A1 mutations, focusing on mutations upstream of the promoter region. The c.-3279T>G mutation is located in the distal upstream region of UGT1A1 (Sugatani et al., 2002). As mentioned above, it has been reported that most Gilbert Syndrome patients with homozygous A(TA)7TAA were also homozygous for c.-3279T>G (Maruo et al., 2004; Costa et al., 2005; Ferraris et al., 2006). This finding suggests a synergistic effect of A(TA)7TAA and c.-3279T>G on transcription activity.

With regard to the prevalence of the UGT1A1 polymorphic mutations in Southeast Asian countries, A (TA)7TAA or c.211G>A were common in Malays (Sutomo et al., 2004; Yusoff et al., 2006). However, the frequency of c.-3279T>G was significantly higher in infants with neonatal jaundice than in control infants. The finding suggests that c.-3279T>G (and some other mutations) is a risk factor for Gilbert Syndrome or neonatal jaundice in Malays (Morioka et al., 2010).

In red blood cells, glucose-6-phosphate dehydrogenase (G6PD) catalyzes NADP to its reduced form, NADPH, in the pentose phosphate pathway (Frank JE, 2005). Limited production of NADPH increases the vulnerability of red blood cells to oxidative stress, which may shorten RBCs life span (Pandolfi et al., 1995).

The main symptom of G6PD deficiency is hemolytic anemia, which usually occurs after exposure to certain medications (antimalarias, primaquine, sulfonamides, nitrofurantoin and other drugs), foods (especially fava beans) or even infection (hepatitis viruses A and B, cytomegaloviruses, pneumonia and others) (Frank, 2005; Beutler, 1996; Cappellini and Foirelli, 2008). Whatever the cause of the acute hemolysis in G6PD deficiency, it is clinically characterized by fatigue, back pain, anemia and jaundice (Frank, 2005; Beutler, 1996). There are approximately 400 million people suffering from G6PD deficiency throughout the world (Cappellini and Foirelli, 2008). The deficiency occurs with high
frequency in Africa, the Mediterranean (including in Italians, Greeks, Arabs and Sephardic Jews), the Middle East and Southeast Asia.

A study conducted in the United States estimated that 30% of jaundiced infants who have permanent brain damage are G6PD deficient (Johnson et al., 2002). However, Kaplan et al., suggested that impaired bilirubin conjugation and delayed clearance by the liver have a considerable contribution to neonatal jaundice (Kaplan et al., 1996; Kaplan et al., 2002a). Based on their clinical data, Jalloh et al., showed that hemolysis is not a main determinant of neonatal jaundice in G6PD deficient infants (Jalloh et al., 2005). Recently, the combined effects of G6PD deficiency and GS on the development of neonatal jaundice have been discussed (Kaplan et al., 1997; Kaplan, 2001a). G6PD deficiency is an X-linked recessive disease (Frank, 2005; Beutler, 1996; Mason et al., 2007). Thus, the disease usually affects males but there are some female patients (Mason et al., 2007). More than 160 different mutations have been demonstrated so far, most of which are missense mutations (Beutler, 1996; Cappellini and Foirelli, 2008; Mason et al., 2007). Most polymorphic mutations predominate in specific regions of the world: G6PD A- (376G/202A) is prevalent in Africa and Southern Europe (Beutler E, 1996 and Mason PJ et al., 2007), G6PD Mediterranean (563T) in Mediterranean countries (Beutler, 1996; Mason et al., 2007), and G6PD Viangchan (871A) in Asian countries (Beutler, 1996; Cappellini and Foirelli, 2008; Mason et al., 2007). Polymorphic mutations affect amino acid residues throughout the enzyme and decrease the stability of the enzyme in the red blood cells, possibly by perturbing protein folding. However, severe mutations mostly affect amino acid residues at the dimer interface or the residues interacting with a structural NADP molecule that stabilizes the enzyme (Mason et al., 2007). Along with other authors have reported that at least 15 different types of single-point mutation are responsible for G6PD deficiency in Taiwanese (Huang et al., 1996; Huang et al., 2002; Tang et al., 1992; Lo et al., 1993; Chen et al., 1997). Most of the G6PD-deficient neonates who had suffered from hyperbilirubinemia carried the mutation at nucleotide 1376 (Huang et al., 1996).

Organic anion transporters (OATs) or organic anion transporting polypeptides (OATPs) play an essential role in the elimination of numerous endogenous and exogenous organic
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Anions from the body. The OAT family is expressed in the kidney, liver, brain and placenta (Anzai et al., 2006). Organic anion transporter 2 (OATP2) is involved in the hepatic uptake of a broad array of endogenous compounds, such as taurocholate, leukotriene C4, prostaglandin E2, conjugated steroid, thyroid hormone and peptide. Recently, OATP2 has also been shown to mediate the cellular uptake of bilirubin and its glucuronide conjugates (Cui et al., 2001).

The OATP2 gene is located in chromosome 12. Huang et al., reported that neonates who carried a polymorphic mutation G>A at nucleotide 388 in the OATP2 gene were at high risk of developing severe hyperbilirubinemia (Huang et al., 2004). According to their data, the prevalence of the variant OATP2 gene was significantly higher in infants with neonatal hyperbilirubinemia than in control infants in the Taiwanese population. This mutation may increase unconjugated bilirubin levels by impairing hepatic bilirubin uptake. Huang et al. also showed that the odds ratio of hyperbilirubinemia in the neonates who carry three risk factors, breast feeding, c.211G>A in UGT1A1 and c.388G>A in OATP2, is 88.00 (95% CI 12.50-642.50, p<0.001). However, Prachukthum et al., reported that there was no association between the variant OATP2 gene and neonatal hyperbilirubinemia in the Thai population: the prevalence of the variant OATP2 gene in infants with hyperbilirubinemia was not higher than in control infants (Prachukthum et al., 2009). It was demonstrated recently that OATP 2 is responsible for the transportation of organic anions into hepatocytes (Cui et al., 2001). This mechanism may also be involved in the transportation of unconjugated bilirubin (Cui et al., 2001). Although variations at nucleotides 388, 463, 521, and 1463 in the OATP 2 gene have been reported (Tirona et al., 2001; Nozawa et al., 2002).

The high incidence of neonatal jaundice in Asian populations compared with Caucasian populations is very likely to have a genetic cause. For many populations, especially in South and Southeast Asia, a lot of further research is needed to elucidate the genetic disorders underlying neonatal jaundice. 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.
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AIMS AND OBJECTIVES

Well established risk factors including presence of polymorphisms/mutations in three genes UGT1A1, OATP 2 and G6PD which are responsible for genesis of hyperbilirubinemia in different ethnic population. Keeping in view the above lacunae, the present study was aimed at “Identification and Molecular Characterization of Mutations and Polymorphism in UGT1A1, OATP 2 and G6PD Genes in Term and Late-Preterm Neonates with Severe Hyperbilirubinemia” which was carried out with the following objectives:

1. Identification of most common mutations (G211A, C686A, C1091T, T1456G) and A(TA)nTAA in UGT1A1 gene, (C188T, C44G) in G6PD gene and (G388A, C463A, T521C and G1463C) in OATP gene of neonates with hyperbilirubinemia.

2. Identification and molecular characterization of unknown/novel mutations in UGT1A1 gene using SSCP and subsequent automated DNA sequencing.

3. Identification and characterization of the polymorphisms in UGT1A1, G-6-PD and OATP genes from hyperbilirubinemic neonates.

4. To establish correlation between genotypes and phenotypes if any.