Mutation analyses of bilirubin UGT1A1 gene in Neonatal that Associated With Hyperbilirubinemia in Al-Najaf province

Rand Muhammed Abdul-Hussain Al-Hussaini
Dept. Laboratory Investigations, Faculty of Sciences, Kufa University.
Correspondence should be sent to: rand.alhussieni@uokufa.edu.iq

Abstract
The objectives of this study were to determine the frequencies of the mutations in the bilirubin UDP glycosyltransferase _UGT1A1 gene which associated with neonatal hyperbilirubinemia in a group of Iraqi babies in Al-Najaf province and a group of normal controls and to compare the frequencies of this mutation between these groups. The newborns with hyperbilirubinemia in this study were 92 newborns with a total bilirubin level of more than 15 mg/dl serum in the first 7 days of life. They showed no blood group and Rh incompatibility, and no clinically detectable pathology except for hyperbilirubinemia. While control group consists from 20 healthy newborns without hyperbilirubinemia all were without clinical manifestation of any disease. This study was carried out in the laboratory of biochemistry in Al-Zahraa Pediatric and Maternal Teaching Hospital and laboratory of molecular biology in the Department of Biology in the Faculty of Sciences – Kufa University, during the period from July 2013 through March 2014.

The distribution of hyperbilirubinemia according to gender showed higher rates in male newborns than female newborns with a significant difference (P < 0.05) between them. The estimated incidence of hyperbilirubinemia increased in the age group (1d-3d) but without a significant difference in comparison with the other groups. The mutation analyses of bilirubin UDP glycosyltransferase _UGT1A1 gene, revealed that the genotypic distribution for G71R mutation among the hyperbilirubinemia group (92) has been found that 11 of 92 newborn had the G71R mutation (heterozygotes), which mean that the UGT1A1 gene mutation was a possible risk factor for the development of neonatal hyperbilirubinemia in Iraq.

Key word: UGT1A1 gene, Neonatal hyperbilirubinemia, Iraq, jaundice, G71R mutation.

Introduction
Severe hyperbilirubinemia is the most common cause of neonatal readmissions for hospitals. Identification of the cause of neonatal hyperbilirubinemia is useful in determining whether therapeutic interventions can prevent severe hyperbilirubinemia [1].

Jaundice is observed during the 1st week of life in approximately 60% of term infants and 80% of preterm infants [2]. The common causes include breastfeeding, dehydration, ABO incompatibility, sepsis, RH incompatibility, Glucose-6-phosphate dehydrogenase (G6PD) deficiency, immature mechanisms for conjugation, and Decreased clearance such as inherited defects in uridine diphosphogluconurate glucuronylsyl-transferase (UGT) [3,4,5,6].

Uridine diphospho glucuronyl-transferase (UGT) is a hepatic enzyme that catalyzes the glucuronization of bilirubin into a water-soluble conjugated form to facilitate in its excretion [7, 8].

UGT is encoded by the UGT1A1 gene at chromosome 2q37.1, whose genotypes with reduced enzyme activity are known to lead to hyperbilirubinemia.
Recent genome-wide association studies showed that serum bilirubin is associated with a genetic variation of the UGT1A1 locus [9]. Mutations in the UGT1A1 gene either reduce the affinity of UGT1A1 toward bilirubin or reduce enzyme activity [10]. The low enzyme activity of UGT may disturb the excretion of conjugated bilirubin and urobilinogen into urine, possibly resulting in a lower frequency of urine bilirubin and urobilinogen under normal conditions [8].

This study was done to investigate the relationship between gene variation and neonatal hyperbilirubinemia and to determine the frequency of UGT1A1 gene mutations in neonatal hyperbilirubinemia by detect genes expression by polymerase chain reaction (PCR) method. Correlate UGT1A1 gene mutations with clinicopathological features (age and sex), and other prognostic parameters (bilirubin concentration) was then done.

Materials and Methods

This study was carried out in the laboratory of biochemistry in Al-Zahra Pediatric and Maternal teaching Hospital and laboratory of molecular biology in the Department of Biology in the Faculty of Sciences – Kufa University, during the period from July 2013 through March 2014.

1. Study and Control Subjects

a) Study group: This study was included ninety-two newborns with hyperbilirubinemia. The definition of newborns with hyperbilirubinemia in this study is ‘newborns with a total bilirubin level of more than 15 mg/dl serum in the first 7 days of life’. All infants were born at 37-42 weeks gestation and weighed more than 2500g (mean =3532 g). They showed no blood group and Rh incompatibility, and no clinically detectable pathology except for hyperbilirubinemia.

b) Control group: consists from 20 healthy newborn without hyperbilirubinemia all were without clinical manifestation of any disease. There were no significant differences between the hyperbilirubinemia and non-hyperbilirubinemia groups in gestational age, and birth weight.

2. Collection of Blood Samples

Two ml of venous blood was collected from clinically suspected patients and control. One ml was allowed to clot at room temperature then centrifuged at 3000 rpm for 5 minutes the serum was used freshly for the Biochemical test of bilirubin. One ml of whole blood was collected in EDTA tubes and store at 20°C until used for PCR test.

3. Billirubin Test

Serum bilirubin estimation was done by Billirubin Kit, which is a product of BIOLABO REAGENT (01260, Maizy, France). The babies who were having serum bilirubin 10 mg/dl or less were not included in the study.

4. DNA isolation and Polymerase Chain Reaction (PCR)

Genomic DNA was isolated using protocol from Genomic DNA Mini Kit was designed specifically for purifying DNA from frozen blood.

A sequence of mutation in the UGT1A1 gene was amplified using this primer-pair:

\[
\begin{align*}
5' & \text{- GACGCCTCGTT GTACATCAGAG} \\
3' & \text{- C} \\
5' & \text{- TCACACGCTGCAGGAAAGAA} \\
3' & \text{- 3'}
\end{align*}
\]

The Sense-primer and antisense-primer were used for codon 71 to do sequence genotyping of the UGT1A1 gene. The sense-primer contains1-bp mismatch
(underlined) immediately proximal to the 3’ end; and the 143-bp amplified product possesses a restriction site for endonuclease MSP1 (synthesized by Promega Corporation, USA, Cat.No. R6401). MSP1 digestion of the PCR amplicon resulted in 119-bp and 24-bp fragments for the wild-type gly71 allele, but failed to cleave the 143-bp fragment containing the Gly71> Arg mutant allele.

PCR was performed using PCR RFLP method. The primers for UGT1A1 gene (synthesized by AccuOligo® Bioneer Corporation .USA) were published previously [11].

Amplification was carried out in 20 µl tube of PCR PreMix Reaction Mixture (PCR PreMix, Bioneer Corporation, USA) containing 5 µl of template DNA (62.5 ng), 1 unit DNA polymerase, 2 µl reaction buffer, 2 µl stabilizer and loading-dye, 2 µl dNTPs, and 2µl of each primer(2 µl forward and 2 µl reverse). Distilled water was added to the final volume of 20 µl.

Amplification was performed in a thermal cycler(Cleaver scientific Ltd/UK) programmed for 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 1 min, preceded by an initial denaturation of 5 min at 95°C. Final extension was for 7 min at 72°C. Finally, the gel electrophoresis method was done according to Sambrook and Russell [12], and 5 µl of each samples was loaded onto 1% agarose gel.

5. Statistical Analysis: Statistical analyses of all results were carried out by the help of SPSS version 17 software statistical package using chi square (P value was considered significant at level less than 0.05).

Results
The study population was included 92 newborns confirmed with hyperbilirubinemia (The diagnosis was under the supervision of pediatrician from the hospital). All infants were born at 37-42 weeks gestation and weighed more than 2500g (mean =3512 g). They showed no blood group and Rh incompatibility, and no clinically detectable pathology except for hyperbilirubinemia.

Distribution of Cases According to Gender:
The distribution of hyperbilirubinemia according to gender showed higher rates in male newborns than female newborns with a significant difference (P < 0.05) between them. The positive cases were 59 (64.13%) males and 33 (35.87%) females (Figure 1).
Figure 1: The Distribution of Newborns with Hyperbilirubinemia Cases According to Gender.

Billirubin concentration according to the gender of patients, the male subjects had a higher level of billirubin as compared to female subjects (15.59% mg/L Vs 20.7% mg/L) and this difference was not significant (P > 0.05) (Figure 2).

Figure 2: Billirubin concentration according to the gender of patients.

Distribution of Cases According to Age:
Assessment of age presentation of hyperbilirubinemic group revealed that 36 (39.13%) patients were seen in age group (1d-3d), 30 (32.6%) in age group (3d-5d), and 26 (28.26%) in age group (5d-7d) (Figure 3). The estimated incidence of hyperbilirubinemia increased in the first age group (1d-3d) but without a significant difference in comparison with the other groups.
Figure 3: Age Distribution of the newborns with Hyperbilirubinemia Cases (Group1: 1d-3d, Group2: 3d-5d, Group3: 5d-7d).

Molecular study (UGT1A1 Gene Mutation)
1. UGT1A1 Gene Mutational Status in healthy newborns:
The control group, 20 healthy newborn without hyperbilirubinemia, has been found as homozygote G/G (100%) (Figure 4).

2. UGT1A1 Gene Mutational Status in hyperbilirubinemia newborns:
The genotypic distribution for G71R mutation among the hyperbilirubinemia group (92) has been found as G/G 81 (88.04%) or wild homozygote, G/R 11 (11.96%) or mutant heterozygote and R/R 0 or mutant homozygotes (Table 1).

Analysis of frequency of the allele carrying mutation (G71R) in codon 71 indicates that the hyperbilirubinemic group is not significantly different from the control group, however the incidence of mutation in hyperbilirubinemic group was higher than the control group with a significant difference (P< 0.05). Analysis of UGT1A1 revealed that hyperbilirubinemia had the identical transition mutation in the codon (GGA to AGA) that caused Arg to replace Gly at position71 (G71R).

Table 1: The Results of the PCR Data with genotypic distribution of G71R mutation.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>PCR results</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>homozygote</td>
<td>heterozygote</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>hyperbilirubinemia</td>
<td>81</td>
<td>11</td>
</tr>
<tr>
<td>group</td>
<td>88.04%</td>
<td>11.96%*</td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td>Total</td>
<td>101</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>90.18%*</td>
<td>9.82%</td>
</tr>
</tbody>
</table>

*P >0.05  significant
Figure 4: PCR amplified 525 bp of UGT1A1 Gene Mutation, 1: (100 bp) DNA ladder; 2,3,4: normal homozygote and 5,heterozygote.

Discussion

Jaundice is an important problem in the first week of life. It is a cause of concern for the physician and a source of anxiety for the parents. High bilirubin levels may be toxic to the developing central nervous system and may cause neurological impairment even in term newborns [13].

The study population was included 92 newborns confirmed with hyperbilirubinemia, their bilirubin was more than 15 mg/dl serum. They needed either intensive phototherapy or exchange transfusion. The incidence and severity of neonatal hyperbilirubinemia is significantly higher in Asians, more so in North Indians, than in Caucasians [14].

The incidence is different among races. The peak serum levels of unconjugated bilirubin in full-term Asian (Japanese, Korean, or Chinese) and American Indian neonates are almost double as those in Caucasian and black populations [15]. The incidence of kernicterus is also higher among Asian newborn infants. These findings suggest that genetic factors are involved in the development of neonatal hyperbilirubinemia [16].

In this study, the distribution of newborns with hyperbilirubinemia according to gender showed higher rates of infection in male than female.

This results agree with another an Iraqi study. One hundred forty newborns with hyperbilirubinemia more than 12.9 mg/dl were studied. Onset of jaundice was mainly on the second and third day of life with a male to female ratio of 1.8:1 [17].

Some studies also suggest that the male child is more likely to have jaundice than a female [18, 19].

The Y-chromosome effect has been postulated to be responsible for these differences. Despite technologic advances in neonatal medicine, reports continue to demonstrate higher rates of morbidity and mortality in males [20, 21].

Dysfunction of the placenta can be a factor, as described in association with male fetus pregnancies [22].

In addition, a higher metabolic rate in the male fetuses may be another contributing factor. This theory is enforced by the fact that XY blastocysts and embryos grow at an accelerated rate when compared with XX chromosome bearers [23].

Assessment of age presentation of hyperbilirubinemic group revealed that the estimated incidence of hyperbilirubinemia increased in the first age group (1d-3d), our findings are consistent with the reports from Mishra et al. [13] who had reported that Over 60% of term newborns develop jaundice by 48-72 hours of age with 5-10% needing intervention for management of hyperbilirubinemia.

The physiological condition of unconjugated hyperbilirubinemia that was seen in 60% of full term neonates during the first week of life is usually related to prematurity of UDPGT enzyme activity. Statistically, our study is unable to confirm that the higher levels of bilirubin are caused by mutations in UGT1A1 gene. Our findings are consistent with the reports from Akaba et al, [24], Maruo et al. [25],

http://www.uokufa.edu.iq/journals/index.php/ajb/index /
http://iasj.net/iasj?func=issues&jId=129&uiLanguage=en
E.mail: biomgzn.sci@uokufa.edu.iq
Yamamoto et al. [26], Sutomo et al. [27] and Dastgerdy et al. [11] have reported that the frequency of the G71R mutation in neonates with severe hyperbilirubinemia was higher than that in neonates without hyperbilirubinemia.

In conclusion, the UGT1A1 gene mutation was a possible risk factor for the development of neonatal hyperbilirubinemia in Iraq. However, it should also be noted that Frequency of G71R mutation in the hyperbilirubinemia group was not significantly more than that in the control group, so we suggest that the factors other than the G71R mutation in the UGT1A1 gene such as environmental or nutritional factors may contribute to the development of neonatal hyperbilirubinemia.

References:
27- Sutomo, R., Talib, N.A., Yusoff, N.M., Van Rostenberghe, H., Sadewa, A.H.,
Screening for G71R mutation of the UGT1A1 gene in the Javanese-

評价基因bilirubin 27- Sutomo, R., Talib, N.A., Yusoff, N.M., Van Rostenberghe, H., Sadewa, A.H.,
Screening for G71R mutation of the UGT1A1 gene in the Javanese-