Neonatal Hyperbilirubinemia and Organic Anion Transporting Polypeptide-2 Gene Mutations

Gökhan Büyükkale, M.D., Ph.D.,[1] Gülcan Turker, Ph.D.,[1] Murat Kasap, M.D.,[2] Gürler Akpınar, M.D.,[2] Engin Arısoy, M.D.,[3] Ayla Günlemez, M.D., Ph.D.,[1,3] and Ayse Gökalp, M.D.[1,3]

ABSTRACT

The aim of this study was to investigate the genotypic distribution of organic anion transporting polypeptide 2 (OATP-2) gene mutations and the relationship with hyperbilirubinemia of unknown etiology. Polymerase chain reaction, restriction fragment length polymorphism, and agarose gel electrophoresis techniques were used for detection of OATP-2 gene mutations in 155 newborn infants: 37 with unexplained hyperbilirubinemia, 65 with explained hyperbilirubinemia, and 53 without hyperbilirubinemia. In the OATP-2 gene, we identified A→G transitions at nucleotide positions 388 and 411 and observed six polymorphic forms. The 388/411–411 mutation was the most common form (43%) in subjects with hyperbilirubinemia of unknown etiology. Male sex [odds ratio (OR): 3.08] and two polymorphic forms of the OATP-2 gene [the 388/411–411 A→G mutation (OR: 3.6) and the 388–411 mutation (OR: 2.4)] increased the risk of neonatal hyperbilirubinemia. In male infants with the 388 A→G mutation of the OATP-2 gene, the levels of unconjugated bilirubin in plasma were significantly increased compared with those observed in females. The polymorphic forms of 388 nucleotide of the OATP-2 gene were identified as risk factors for hyperbilirubinemia of unknown etiology.

KEYWORDS: Newborn, organic anion transporting polypeptide 2 gene, polymorphism, nonphysiological hyperbilirubinemia

Despite advanced therapeutic measures, the overall morbidity of neonatal jaundice remains high, especially newborns in developing countries where acute bilirubin encephalopathy is a serious endemic problem.[1] Hyperbilirubinemia is also a major health problem in Turkey, where the incidence of nonphysiological neonatal hyperbilirubinemia varies between 10.5 and 25.3%.[2] However, in half of all cases, no risk factors associated with nonphysiological hyperbilirubinemia can be identified. As the field of molecular medicine progressed over the last decade, increasing attention was focused on the role of genetic factors in the development of severe hyperbilirubinemia and kernicterus.[3] However, the relationship between hyperbilirubinemia and genetic factors remains unclear.[3,4]

Risk factors for neonatal hyperbilirubinemia include East Asian descent (particularly Japanese, Korean, or Chinese descent), a sibling who was affected by

Departments of [1]Neonatology, [2]Medical Biology, and [3]Pediatrics, Kocaeli University, Kocaeli, Turkey.

Address for correspondence and reprint requests: Gülcan Turker, Department of Neonatology, Kocaeli University, Kocaeli 41380, Turkey (e-mail: gulcanturker@kocaeli.edu.tr).

Am J Perinatol 2011;28:619–626. Copyright © 2011 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA. Tel: +1(212) 584-4662.

Received: November 26, 2010. Accepted after revision: February 25, 2011. Published online: April 15, 2011.
DOI: http://dx.doi.org/10.1055/s-0031-1276736.
ISSN 0735-1631.

Downloaded by: Kocaeli University. Copyrighted material.

hyperbilirubinemia in the neonatal period, and a family history that suggests an underlying genetic cause. An increasing number of studies have focused on the genetics of bilirubin; these studies have sought to detect genetic mutations that lead to elevated unconjugated serum bilirubin, such as those that affect the molecular structure of 5′-diphosphate glucuronosyltransferase (UGT) and those that result in glucose-6-phosphate dehydrogenase (G6PD) deficiency.[5–9] G6PD deficiency is the most common genetic defect around the world, including Turkey.[4] However, few studies have investigated the role of organic anion transporting polypeptide (OATP)-2 in neonatal hyperbilirubinemia.[9,10] The human OATP family consists of 11 members: OATP1A2, -1B1, -1B3, -1C1, -2A1, -2B1, -3A1, -4A1, -4C1, -5A1, and -6A1. OATPs are encoded by genes of the SLCO family (previously SLC21). The genes encoding human OATP1 family members are located in the short arm of chromosome 12 and consist of 2703 nucleotides and 14 exons, whereas genes encoding other OATPs are located in chromosomes 3, 5, 8, 11, 15, and 20. The human OATP2 (also known as human liver-specific organic anion transporter or OATP-C or OATP1B1; the symbol of gen: SLC21A6) showed uptake of monoglucuronosyl bilirubin, bisglucuronosyl bilirubin, and sulfobromophthalein.[11–13] OATPs facilitate sodium-independent uptake transport of a wide variety of organic endo- and exogenous compounds, such as bile acids, bilirubin, steroid, and thyroid hormones and their conjugates, and numerous drugs and toxins.[11–13] Severe hyperbilirubinemia still occurs in Turkish neonates, and it is important to understand the genetic risk factors for the underlying causes of the disease.[14–16] In this study, the role of OATP-2 in neonatal hyperbilirubinemia with unknown etiology was investigated and compared in neonatal hyperbilirubinemia with known etiology. To the best of our knowledge, this is the first study to assess the OATP-2 gene in a case-control analysis of neonatal hyperbilirubinemia in Turkey.

METHODS

The study was approved by the Kocaeli University Faculty of Medicine ethics committee, and written informed consent was obtained from the parents of infants included in the study.

Selection of Study and Control Groups

A total of 207 healthy newborns with gestational ages ≥ 37 weeks were delivered in the Kocaeli University Hospital during the study period. Sick infants who were transferred to the intensive care unit or sick baby nursery were excluded. Newborns with hyperbilirubinemia who had no problems other than hyperbilirubinemia were selected from either the nursery or the neonatal outpatient clinic. All newborns were observed for hyperbilirubinemia for first week according to the guidelines developed by the American Academy of Pediatrics until stabilization of hyperbilirubinemia.[17] The patients were examined every 48 hours in the first 10 days of life, after which controls were performed weekly for prolonged hyperbilirubinemia until stabilization of the jaundice both as inpatients and outpatients. During follow-up visits, all infants received complete physical examinations, their medical histories were briefly recorded, and transcutaneous bilirubin measurements were taken using a BiliCheck device (American Laubscher Corporation, Farmingdale, NY). If the transcutaneous bilirubin level was over 5 mg/dL, total serum bilirubin was rechecked for confirmation. Nonphysiological hyperbilirubinemia was defined as having ≥ 1 mg/dL of serum total bilirubin above the 95th percentile value (high-risk zone).[17] All newborns with hyperbilirubinemia were investigated for the following reasons: congenital anomalies, increased hepatic enzyme levels, congenital hypothyroidism, sepsis, urinary tract infections, insufficient feeding ($>10\%$ loss of birth weight), dehydration, blood extravasations, a history of hypoxia, polycythemia or a metabolic disease, ABO or Rh incompatibility, G6PD enzyme deficiency, or a diabetic mother.

Healthy newborns followed for 4 weeks after birth for nonphysiological hyperbilirubinemia were enrolled as controls.

1. Nonphysiological hyperbilirubinemia of unknown etiology (group I): This group included 65 newborn infants with gestational ages of ≥ 37 weeks and ages of < 10 days. Patients were excluded for the following reasons: congenital anomalies, increased hepatic enzyme levels, congenital hypothyroidism, sepsis, urinary tract infections, insufficient feeding ($>10\%$ loss of birth weight), dehydration, blood extravasations, a history of hypoxia, polycythemia or a metabolic disease, ABO or Rh incompatibility, G6PD enzyme deficiency, prolonged hyperbilirubinemia or insufficient feeding ($>10\%$ loss of birth weight), dehydration, or a diabetic mother.
2. Nonphysiological hyperbilirubinemia of known etiology with hemolytic or blood extravasations (group II): This group included 37 newborns. In this group, the causes of the hyperbilirubinemia were identified as only ABO incompatibility ($n=24$), only Rh incompatibility ($n=7$), ABO and Rh incompatibility ($n=2$), G6PD enzyme deficiency ($n=2$), cephalhematoma ($n=1$), or adrenal hemorrhage ($n=1$). Patients were excluded for the following reasons: congenital anomalies, increased hepatic enzyme levels, congenital hypothyroidism, sepsis, urinary tract infections, insufficient feeding ($>10\%$ loss of birth weight), dehydration, a history of hypoxia, polycythemia or a metabolic disease, or a diabetic mother.

Downloaded by: Kocaeli University. Copyrighted material.

3. Control group (group III): This group included 53 healthy full-term newborns. Control subjects had maximum value of total bilirubin levels < 40th percentile (low-risk zone) during the study period.[17] If infants with prolonged or nonphysiological hyperbilirubinemia for 1 month were excluded from the control group. Prolonged hyperbilirubinemia was identified in infants who had serum total bilirubin (STB) levels of 150 μmol/L (8.8 mg/dL) or more for 30 days. Newborns were observed for jaundice, and STB concentrations were measured when visible jaundice was noticed, both as inpatients and outpatients, until stabilization of the jaundice. Forty-five newborns were excluded from the study and control groups because of prolonged jaundice or other characteristics. Eleven infants with insufficient feeding jaundice were excluded. The following information was recorded for each subject: name, gestational age, birth weight, blood group, direct Coombs test result, reticulocyte count, hemogram and peripheral smear results, hepatic function test results, family history, and therapeutic methods (i.e., phototherapy, exchange transfusion, and phenobarbital use).

Total serum bilirubin levels were measured by a spectrophotometric method (B-105 Digital bilirubinometer Erma, Inc., Japan). G6PD enzyme levels were analyzed quantitatively by a UV kinetic measurement method with Trinity Biotech test kits (County Wicklow, Ireland) in the Cobas Mira chemical analyzer. Newborns with G6PD levels less than 4.6 U/g/Hb were considered to have a G6PD deficiency. ABO incompatibility was defined if the mother's blood type was O and her infant's blood type was A or B. Direct Coombs tests were performed by gel centrifugation, a sensitive technique for identifying immunoglobulin G-coated red blood cells.

Genetic Analysis

Blood samples (2 to 3 mL) were collected in tubes containing ethylenediaminetetraacetic acid to investigate the etiology of the hyperbilirubinemia. Genomic DNA was isolated using the ammonium acetate method.[18] DNA samples were kept at 4°C for short-term storage. The 25-μL polymerase chain reaction (PCR) mixtures consisted of 1 × PCR buffer, 0.2 mmol/L of each deoxyribonucleotide triphosphate, 1.0 mmol/L of each primer, 1.25 mmol/L $MgCl_2$, 1.0 U of Taq polymerase, and 100 ng of genomic DNA. An initial 5-minute denaturation at 94°C was followed by 35 cycles consisting of 30 seconds of denaturation at 94°C, 1 minute of annealing at 55°C, and 1 minute of elongation at 72°C. PCR reactions ended with a 10-minute final elongation at 72°C. PCR products were analyzed with agarose or polyacrylamide gel electrophoresis. PCR products were cleaned with a PCR purification kit (Qiagen, Venlo, The Netherlands) and sequenced when necessary (Iontek Inc., Istanbul, Turkey).

For OATP-2, the sense and antisense primers used were 5′-ATAATGGTGCAAATAAAGGGG-3′ and 5′-ACTATCTCAGGTGATGCTCTA-3′, respectively. The OATP-2 PCR products were cleaved with *Taq*I under recommended conditions (Fermentas Inc., Glen Burnie, MD). The cleavage products were analyzed by polyacrylamide gel electrophoresis, and the bands were visualized with silver nitrate staining. The final fragment size was 214 bp.

RESULTS

The descriptive characteristics of the three groups are summarized in Table 1. Differences in birth weight and gestational age among the three groups were not statistically significant. Turkey's post hoc statistical analysis showed that there were significantly more males than females in group I ($p < 0.0001$; Table 1). The total serum bilirubin levels in group II were statistically higher than those in group I ($p < 0.023$). There was not a statistically significant difference in the use of phototherapy treatments between group I and group II ($p = 0.4$). Exchange transfusion was not performed on any members of group

Table 1 Descriptive Characteristics of Subjects with Nonphysiological Hyperbilirubinemia

	Group I ($n = 65$)	Group II ($n = 37$)	Group III ($n = 53$)	p
Sex*				
Female, *n* (%)	24 (36.9)	18 (48.7)	37 (69.8)	0.002
Male, *n* (%)	41 (63.1)	19 (51.3)	16 (30.2)	
Birth weight (g) (mean ± standard deviation)†	3156 ± 569	3233 ± 496	3248 ± 412	0.573
Gestational age (wk)†	38 ± 1.7	38 ± 1.5	38 ± 1.6	0.765
Peak total bilirubin level (mg/dL)‡	21 ± 4,1	23.6 ± 5.8	4.6 ± 0.8	0.023
G6PD	0	2	0	—
Phototherapy, *n* (%)*	60 (92)	35 (94.5)	—	—
Exchange transfusion, *n* (%)*	0	3 (8.1)		0.6

*Chi-square tests were used for statistical analysis.
†Kruskal-Wallis.
‡Mann-Whitney *U*.
Group I, subjects with nonphysiological hyperbilirubinemia of unknown etiology; group II, subjects with known etiology; group III, control group.

Downloaded by: Kocaeli University. Copyrighted material.

Table 2 The Distribution of OATP-2 Gene Mutations in the Three Groups of Newborns

	OATP-2 Gene AG Mutations					
	388–388 Mutation, *n* (%)	**388–388/411 Mutation, *n* (%)**	**388–411 Mutation, *n* (%)**	**388–388/411 Mutation, *n* (%)**	**388/411–411 Mutation, *n* (%)**	**411–411 AG Mutation, *n* (%)**
Group I	1 (1.5)	3 (4.6)	15 (23.0)	4 (6.1)	28 (43.0)	14 (21.5)
Group II	1 (2.7)	3 (8.1)	5 (13.5)	4 (10.8)	8 (21.6)	16 (43.2)
Group III	2 (3.7)	4 (7.5)	10 (18.8)	6 (11.3)	14 (26.4)	17 (32.0)
Total	4 (2.5)	10 (6.4)	30 (19.3)	14 (9.0)	50 (32.2)	47 (30.3)

Group I, subjects with nonphysiological hyperbilirubinemia of unknown etiology; group II, subjects with nonphysiological hyperbilirubinemia of known etiology; group III, control group. OATP-2, organic anion transporter 2.

I, although three female newborns in group II received exchange transfusion. These three newborns who received exchange transfusion were diagnosed with ABO incompatibility. Exchange transfusion was performed because their total bilirubin levels were 2 mg/dL above the threshold for exchange transfusion. The difference in the exchange transfusion rates of groups I and II was not significant ($p = 0.13$; Table 2).

OATP-2 Polymorphisms

A→G single nucleotide transitions were observed at nucleotide positions 388 and 411 of the OATP-2 gene; these changes created cut sites for *Taq*I (Fig. 1). If a transition occurred at nucleotide position 388 but not at nucleotide position 411, two fragments of 128 and 86 bp were expected to be generated. If a transition occurred at nucleotide position 411 but not at nucleotide position 388, two fragments of 151 and 63 bp were expected to be generated. If transitions occurred at nucleotide positions 388 and 411, three fragments of 128, 86, and 63 bp were expected to be generated (Fig. 1A). Analysis of the polyacrylamide gels confirmed these different DNA fragmentation patterns for the OATP-2 gene as six polymorphic forms (Fig. 1B). The six different DNA fragmentation patterns were as follows: only homozygous 388 (388–388) A→G mutations; homozygous 388 and heterozygous 411 (388–388/411) A→G mutation; homozygous 388 and 411 (388–388/411–411) A→G mutation; heterozygous 388 and 411 (388/411) A→G mutation; heterozygous 388 and homozygous 411 (388/411–411) A→G mutation; and homozygous 411 (411–411) A→G mutations (Figs. 1A and 1B). In group I, DNA fragmentation patterns showed that the 388/411–411 mutation of the OATP-2 gene was most common; in groups II and III, the homozygous 411 (411–411) A→G mutation of the OATP-2 gene was most common (Table 2). The 388 A→G mutation of the OATP-2 gene significantly increased in both female and male infants (approximately threefold) but in infants with this mutation, total bilirubin levels significantly increased in male compared with female (as median; female: 16.1 mg/dL, male: 19.1 mg/dL, $p = 0.02$).

We used forward stepwise logistic regression analysis to determine the risk factors for nonphysiological hyperbilirubinemia of unknown etiology. We entered birth weight, the six different polymorphic forms of the OATP-2 gene, sex, and G6PD enzyme deficiency as risk factors into the logistic regression program. Only the 388/411–411 and the 388–411 A→G mutations of the OATP-2 gene as well as sex were identified as risk factors for hyperbilirubinemia of unknown etiology (Table 3). Birth weight and other observed polymorphic forms of OATP-2 were not identified as risk factors for neonatal hyperbilirubinemia of unknown etiology (Table 3).

Male sex increased the risk of neonatal nonphysiological hyperbilirubinemia of unknown etiology by a factor of 3.08 (odds ratio [OR]), the 388/411–411 A-G mutation of the OATP-2 gene increased the risk by a factor of 3.6 (OR), and the 388–411 A-G mutation of the gene increased the risk by a factor of 2.4 (OR).

DISCUSSION

Despite the extensive use of phototherapy, exchange transfusion, and maternal anti-D immunoglobulin (Rhogam, Ortho-Clinical Diagnostic, Inc., Rochester, NY), kernicterus still occurs; these cases highlight the need for continued study of the etiology of hyperbilirubinemia.[19] Neither hyperbilirubinemia nor kernicterus are reportable diseases, and there are no reliable sources of information providing national annual estimates.[19] The primary risk factors include blood group incompatibility, maternal factors (such as diabetes or drug use), birth trauma, breast-feeding, weight loss, premature birth, polycythemia, and ethnicity. However, these risk factors are present in only ~50% newborns with nonphysiological hyperbilirubinemia, thus further investigations are required to establish the causes of this condition.[19] Nonphysiological hyperbilirubinemia is more evident and frequent in children from Eastern Mediterranean and Asian groups than in those from black and white ethnic groups.[1,3,4,9,11,19,20] Nonphysiological hyperbilirubinemia with unknown etiology among Turkish newborns is prevalent, suggestive of genetic risk in this population. In our country, G6PD

Downloaded by: Kocaeli University. Copyrighted material.

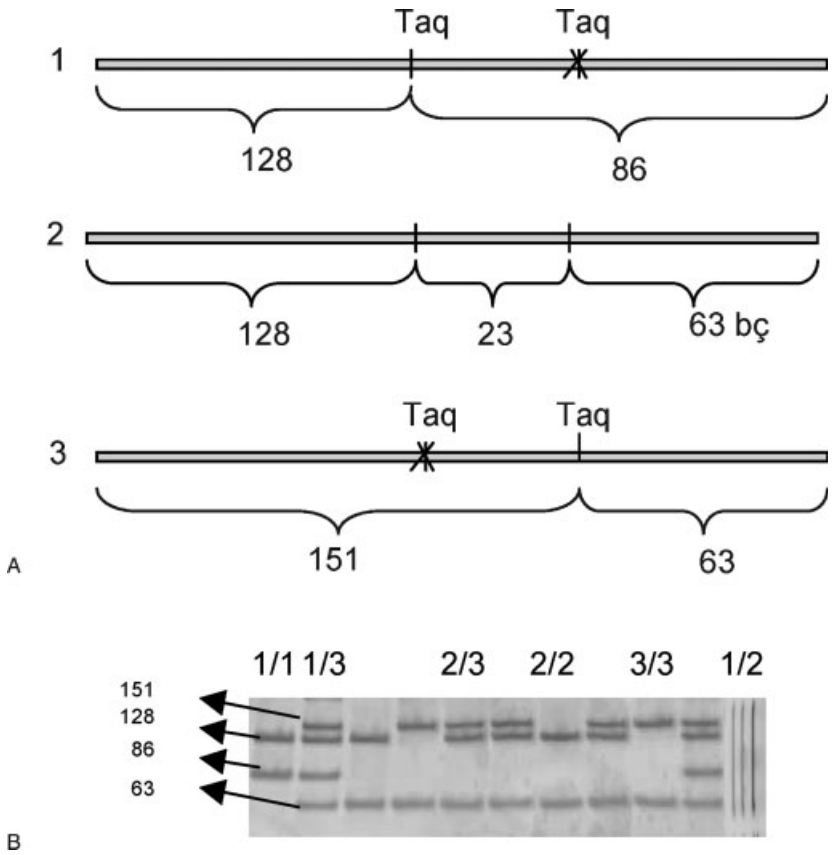


Figure 1 The bands for the polymorphic forms of the organic anion transporting polypeptide 2 (OATP-2) gene after *Taq*I digestion were detected on a polyacrylamide gel. (A) In silico analysis of expected fragmentation patterns for the polymorphic forms of OATP-2 gene after *Taq*I digestion. (B) A silver-stained polyacrylamide gel showing the observed polymorphic band patterns. The polymorphic form 1/1 is a homozygotic form in which the OATP-2 gene was cut at base number 388. The polymorphic form 1/2 is a heterozygotic form with a cut site at base 388 on both alleles and at base 411 on one allele. The polymorphic form 1/3 is a heterozygotic form with a cut site at base 388 on one allele and at base 411 on the other allele. The polymorphic form 2/2 is a homozygotic form with cut sites at bases 388 and 411 on both alleles. The polymorphic form 2/3 is a heterozygotic form with cut sites at bases 388 and 411 on one allele and at base 411 on the other allele. The polymorphic form 3/3 is a homozygotic form with a cut site at base 411 on both alleles. The 23-bp band was not within the resolution limits of the polyacrylamide gel used in this study.

Table 3 Risk Factors for Nonphysiological Hyperbilirubinemia of Unknown Etiology

	B	OR (95% CI)	*p*
OATP-2 388/411–411 mutation	1.28	3.6 (1.63–7.96)	0.0014
OATP-2 388–411 mutation	0.9	2.4 (1.008–6.16)	0.048
Gender	1.1	3.08 (1.53–6.19)	0.0016
Constant	−2.64		0.0000

Constant: nonphysiological hyperbilirubinemia of unknown etiology. Other variables were glucuronosyl transferase enzyme deficiency, other 388–388 A→G mutation; 388–388/411–411 A→G mutation; 388/411–411 A→G mutation; and, 411–411 A→G mutation. OATP-2, organic anion transporter 2; B, estimate of the change in the dependent variable that can be attributed to a change of one unit in the independent variable; CI, confidence interval; OR, odds ratio.

deficiency and UGT1A1 gene polymorphisms have been investigated in many studies.[14–16,21–23] To date, however, no study has investigated mutations in OATP-2. This study also investigated the role of OATP-2 gene polymorphisms in neonatal hyperbilirubinemia.

G6PD deficiency and mutation is another genetic risk factor causing neonatal hyperbilirubinemia. In this study, G6PD deficiency was present in 2% of newborns with hyperbilirubinemia. There has been no more information about the ratio of G6PD deficiency in Kocaeli region. But alternatively, several studies have examined these mutations in another region of Turkey. Atay et al[21] found that the frequency of G6PD deficiency in subjects with increased indirect bilirubin levels was 3.8%, and Kocabay et al[22] found that this frequency varied from 1

Downloaded by: Kocaeli University. Copyrighted material.

to 5%. Keskin et al[23] reported the frequency of G6PD deficiency as 1.2% in their subjects, and the frequency of the 563 C→T mutations in the G6PD Mediterranean gene was 79% in their patients.[23] False-negative results with biochemical methods may have been obtained by these investigators due to their methods, which were designed for diagnosis of G6PD deficiency. Or the cutoff point can be set by following the appropriate instructions and by trial and error in the individual diagnostic laboratory.[24] Luzzatto and Poggi reported that ideally, every patient found to be G6PD deficient by screening should be confirmed by the spectrophotometric assay.[24] But we did not confirm by the spectrophotometric assay. Neonatal hyperbilirubinemia is more common in male infants.[17] Our results confirm the findings of previous studies in Turkey, which indicated that 63.1% of newborns with nonphysiological hyperbilirubinemia of unknown etiology and 51.3% of newborns with nonphysiological hyperbilirubinemia of known etiology are male.[17,19,22] Heterozygotes have been shown to have a high incidence of hyperbilirubinemia and even kernicterus. Male gender may be considered a risk factor for neonatal hyperbilirubinemia due to the false-negativity of G6PD deficiency. We believe that during the hyperbilirubinemia period, enzyme levels studied with biochemical methods may give false-negative results, and tests should thus be repeated. Based on these results, we conclude that G6PD deficiency mutations must be studied in the Turkish population.

Polymorphisms in the UGT1A1 gene may also be involved in neonatal hyperbilirubinemia. The UGT1A1 gene, located on chromosome 2 (2q37), is composed of 1,599 nucleotides.[25–28] The A[TA]$_7$TAA variant, resulting from a mutation in the promoter region of the UGT1A1 gene, is less common in Asians than in whites. The A[TA]$_7$TAA variant is observed at a rate between 10% and 16.8% in Japanese, 16.2% in Chinese, 18.8% in Malaysian, and 14.3% in Taiwanese subjects; its rate is higher in whites (between 35.7% and 41.5%) and Indians (35.1%).[25–28] In Turkey, Kılıc et al[14] reported a homozygotic A [TA]$_7$TAA frequency of 24.3% in the UGT1A1 gene and a heterozygotic A[TA]$_7$TAA frequency of 10%. Other Turkish researchers have reported values of other polymorphisms in the UGT1A1 gene between 5% and 56%.[29,30] But we did not study any mutation in the UGT1A1 gene; therefore, we have no knowledge about the role of this gene in our population.

Mutations have been identified at nucleotides 388, 463, 521, and 1463 of the OATP2 (SLC21A6) gene (located in the short arm of chromosome 12).[11] In previous studies, only the G→A base variation (D130N) at nucleotide 388 was shown to significantly increase the risk of nonphysiological unconjugated hyperbilirubinemia.[9,31–34] But Wong et al found that variants of OATP-2 gene at nucleotide 388 were not significant risk factors associated with severe unconjugated hyperbilirubinemia in Malaysian Chinese infants.[35] High-affinity uptake of unconjugated bilirubin by OATP2 occurred in the presence of albumin.[11,12] In vitro, OATP2 has been shown to transport both unconjugated and conjugated bilirubin.[11,12] Also Cui et al showed that bilirubin bound to albumin is taken up across the basolateral membrane by OATP2 and conjugated in the hepatocyte by the UGT1A1, resulting in monoglucuronosyl bilirubin and bisglucuronosyl bilirubin. Bilirubin glucuronides are finally excreted into bile by the apical conjugate export pump multidrug resistance protein 2 localized to the hepatocyte canalicular (apical) membrane.[11,12] The differentiation between carrier-mediated and diffusional bilirubin uptake into the liver will be supported by the identification of mutations in the OATP2 (SLC21A6) gene leading to the loss or functional impairment of OATP2 in the basolateral membrane of hepatocytes.[11,12] Van de Steeg et al found marked conjugated hyperbilirubinemia in mice with the mutation of OATP genes. OATP transporters play an essential role in hepatic reuptake of conjugated bilirubin and uptake of unconjugated bile acids and drugs.[36] Therefore, van de Steeg et al hypothesized that substantial sinusoidal secretion and subsequent OATP transporter-mediated reuptake of glucuronidated compounds can occur in hepatocytes under physiological conditions. However, in mice, five different members of the OATP subfamily are controlled only by this gene, but in humans, only one member of OATP subfamily is controlled by this gene. Therefore, we may suggest that the cause of marked conjugated bilirubin may be due to functional deficiency of all five members of OATP subfamily in mice. Also we found that in males with the 388 A→G mutation of OATP-2, unconjugated bilirubin in plasma was significantly increased compared with females who have the same mutation. van de Steeg et al found that in male mice with OATP-2 mutation, unconjugated bilirubin in plasma was significantly increased, compared with female mice as in our study. Therefore, we may suggest that this mutation may affect unconjugated bilirubin levels in male. Maybe the reason for increased the risk of neonatal hyperbilirubinemia in males is the clinical presentation of OATP-2 gene mutation in males as the clinical presentation of G6PD gene mutation. Moreover, in view of the fact that current knowledge of the human OATP family is not complete, additional transport proteins may further contribute to the selective uptake of bilirubin from the blood circulation into liver as suggested by Cui et al and Van de Steeg et al.[12,36]

In the present study, we used the *Taq*I restriction enzyme to obtain three PCR fragments and six polymorphic forms of the OATP-2 gene (Fig. 1). Although Huang et al[9] showed that the 411 A→G restriction site was not polymorphic, we have demonstrated that this

Downloaded by: Kocaeli University. Copyrighted material.

site carries polymorphic properties. The 388/411–411 mutation of the OATP-2 gene was most common among newborns with hyperbilirubinemia of unknown etiology. The homozygous 411 A→G mutation of the OATP-2 gene occurred in 43.2% of newborns with jaundice of known etiology. In addition to male sex, the 388/411–411 and 388–411 mutation forms of the OATP-2 gene were statistically identified as risk factors for hyperbilirubinemia of unknown etiology. Male sex increased the risk of neonatal hyperbilirubinemia by a factor of 3.08, the 388/411–411 mutation of the OATP-2 gene increased the risk by a factor of 3.6, and the 388–411 mutation of the OATP-2 gene increased the risk by a factor of 2.4.

CONCLUSION

Male sex and two polymorphic forms of the OATP-2 gene (388/411–411 and 388–411) increased the risk of neonatal nonphysiological hyperbilirubinemia. In males with the 388 A→G mutation of OATP-2 gene, unconjugated bilirubin in plasma was significantly increased compared with females. Therefore, we suggest that this mutation may affect unconjugated bilirubin levels in males. Further studies are needed to better understand the genetics of jaundice and how to avoid the sequel associated with neonatal nonphysiological hyperbilirubinemia.

REFERENCES

1. Blackmon LR, Fanaroff AA, Raju TNK; National Institute of Child Health and Human Development. Research on prevention of bilirubin-induced brain injury and kernicterus: National Institute of Child Health and Human Development conference executive summary. 2003. Pediatrics 2004;114: 229–233
2. Sarıcı SU, Serdar MA, Korkmaz A, et al. Incidence, course, and prediction of hyperbilirubinemia in near-term and term newborns. Pediatrics 2004;113:775–780
3. Watchko JF, Daood MJ, Biniwale M. Understanding neonatal hyperbilirubinaemia in the era of genomics. Semin Neonatol 2002;7:143–152
4. Watchko JF, Lin Z, Clark RH, Kelleher AS, Walker MV, Spitzer AR. Complex multifactorial nature of significant hyperbilirubinemia in neonates. Pediatric Hyperbilirubinemia Study Group. Pediatrics 2009; 124 e 868–877. Available at: http://pediatrics.aappublications.org/cgi/content/full/124/5/e868 (accessed January 20, 2010)
5. Ritter JK, Crawford JM, Owens IS. Cloning of two human liver bilirubin UDP-glucuronosyltransferase cDNAs with expression in COS-1 cells. J Biol Chem 1991;266:1043–1047
6. Bosma PJ, Chowdhury JR, Huang TJ, et al. Mechanisms of inherited deficiencies of multiple UDP-glucuronosyltransferase isoforms in two patients with Crigler-Najjar syndrome, type I. FASEB J 1992;6:2859–2863
7. Bosma PJ, Chowdhury JR, Bakker C, et al. The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. N Engl J Med 1995;333: 1171–1175
8. Agrawal SK, Kumar P, Rathi R, et al. UGT1A1 gene polymorphisms in North Indian neonates presenting with unconjugated hyperbilirubinemia. Pediatr Res 2009;65:675–680
9. Huang MJ, Kua KE, Teng HC, Tang KS, Weng HW, Huang CS. Risk factors for severe hyperbilirubinemia in neonates. Pediatr Res 2004;56:682–689
10. Prachukthum S, Nunnarumit P, Pienvichit P, et al. Genetic polymorphisms in Thai neonates with hyperbilirubinemia. Acta Paediatr 2009;98:1106–1110
11. Kalliokoski A, Niemi M. Impact of OATP transporters on pharmacokinetics. Br J Pharmacol 2009;158:693–705
12. Cui Y, König J, Leier I, Buchholz U, Keppler D. Hepatic uptake of bilirubin and its conjugates by the human organic anion transporter SLC21A6. J Biol Chem 2001;276:9626–9630
13. Niemi M. Role of OATP transporters in the disposition of drugs. Pharmacogenomics 2007;8:787–802
14. Kılıc I, Cakaloz I, Atalay E. Frequency of UDP-glucuronosyltransferase 1 (UGT1A1) gene promoter polymorphisms in neonates with prolonged and pathological jaundice in the Denizli region of Turkey. Int J Clin Pharmacol Ther 2007;45:475–476
15. Ulgenalp A, Duman N, Schaefer FV, et al. Analyses of polymorphism for UGT1*1 exon 1 promoter in neonates with pathologic and prolonged jaundice. Biol Neonate 2003; 83:258–262
16. Muslu N, Turhan AB, Eskandari G, et al. The frequency of UDP-glucuronosyltransferase 1A1 promoter region (TA)7 polymorphism in newborns and it's relation with jaundice. J Trop Pediatr 2007;53:64–68
17. American Academy of Pediatrics Subcommittee on Hyperbilirubinemia. Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. Pediatrics 2004;114:297–316
18. Sambrook J, Russell DW. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001
19. Ip S, Chung M, Kulig J, et al; American Academy of Pediatrics Subcommittee on Hyperbilirubinemia. An evidence-based review of important issues concerning neonatal hyperbilirubinemia. Pediatrics 2004;114:e130–e153
20. Maruo Y, Nishizawa K, Sato H, Sawa H, Shimada M. Prolonged unconjugated hyperbilirubinemia associated with breast milk and mutations of the bilirubin uridine diphosphate-glucuronosyltransferase gene. Pediatrics 2000;106:E59. Available at: http://pediatrics.aappublications.org/cgi/content/full/106/5/e59 (accessed December 20, 2010)
21. Atay E, Bozaykut A, Ipek IO. Glucose-6-phosphate dehydrogenase deficiency in neonatal indirect hyperbilirubinemia. J Trop Pediatr 2006;52:56–58
22. Kocabay K, Yılmaz S, İlhan N, et al. The investigation of glucose-6-phosphate dehydrogenase deficiency in newborn hyperbilirubinemia with unknown etiology. Turk J Med Res 1995;13:194–198
23. Keskin N, Ozdes I, Keskin A, Acikbas I, Bagci H. Incidence and molecular analysis of glucose-6-phosphate dehydrogenase deficiency in the province of Denizli, Turkey. Med Sci Monit 2002;8:CR453–CR456
24. Luzzatto L, Poggi V. G6PD deficiency. In: Orkin S, Nathan DG, Ginsburg D, Look AT, Fisher DE, Lux SE eds. Nathan

Downloaded by: Kocaeli University. Copyrighted material.

and Oski's Hematology of Infancy and Childhood. Philadelphia: Saunders Elsevier; 2007:883–907
25. Huang CS, Chang PF, Huang MJ, Chen ES, Chen WC. Glucose-6-phosphate dehydrogenase deficiency, the UDP-glucuronosyl transferase 1A1 gene, and neonatal hyperbilirubinemia. Gastroenterology 2002;123:127–133
26. Kadakol A, Ghosh SS, Sappal BS, Sharma G, Chowdhury JR, Chowdhury NR. Genetic lesions of bilirubin uridine-diphosphoglucuronate glucuronosyltransferase (UGT1A1) causing Crigler-Najjar and Gilbert syndromes: correlation of genotype to phenotype. Hum Mutat 2000;16:297–306
27. Kaplan M, Hammerman C, Maisels MJ. Bilirubin genetics for the nongeneticist: hereditary defects of neonatal bilirubin conjugation. Pediatrics 2003;111(4 Pt 1):886–893
28. Monaghan G, Ryan M, Seddon R, Hume R, Burchell B. Genetic variation in bilirubin UPD-glucuronosyltransferase gene promoter and Gilbert's syndrome. Lancet 1996;347: 578–581
29. Ergin H, Bican M, Atalay OE. A causal relationship between UDP-glucuronosyltransferase 1A1 promoter polymorphism and idiopathic hyperbilirubinemia in Turkish newborns. Turk J Pediatr 2010;52:28–34
30. Narter F, Can G, Ergen A, Isbir T, Ince Z, Çoban A. Neonatal hyperbilirubinemia and G71R mutation of the UGT1A1 gene in Turkish patients. J Matern Fetal Neonatal Med 2011;24:313–316
31. Tirona RG, Leake BF, Merino G, Kim RB. Polymorphisms in OATP-C: identification of multiple allelic variants associated with altered transport activity among European- and African-Americans. J Biol Chem 2001;276:35669–35675
32. Tamai I, Nezu J, Uchino H, et al. Molecular identification and characterization of novel members of the human organic anion transporter (OATP) family. Biochem Biophys Res Commun 2000;273:251–260
33. Nozawa T, Nakajima M, Tamai I, et al. Genetic polymorphisms of human organic anion transporters OATP-C (SLC21A6) and OATP-B (SLC21A9): allele frequencies in the Japanese population and functional analysis. J Pharmacol Exp Ther 2002;302:804–813
34. Nishizato Y, Ieiri I, Suzuki H, et al. Polymorphisms of OATP-C (SLC21A6) and OAT3 (SLC22A8) genes: consequences for pravastatin pharmacokinetics. Clin Pharmacol Ther 2003;73:554–565
35. Wong FL, Boo NY, Ainoon O, Wang MK. Variants of organic anion transporter polypeptide 2 gene are not risk factors associated with severe neonatal hyperbilirubinemia. Malays J Pathol 2009;31:99–104
36. van de Steeg E, Wagenaar E, van der Kruijssen CM, et al. Organic anion transporting polypeptide 1a/1b-knockout mice provide insights into hepatic handling of bilirubin, bile acids, and drugs. J Clin Invest 2010;120:2942–2952

Downloaded by: Kocaeli University. Copyrighted material.