

A causal relationship between UDP-glucuronosyltransferase 1A1 promoter polymorphism and idiopathic hyperbilirubinemia in Turkish newborns

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The etiology of pathological jaundice can not be identified in almost half of the cases. The effect of promoter polymorphism in the UDP-glucuronosyltransferase 1A1 (UGT1A1) gene was investigated in healthy breast-fed Turkish neonates with unexplained and direct Coombs'-negative ABO incompatible hyperbilirubinemia. Newborns whose peak serum bilirubin levels were ≥ 17 mg/dl and ≤ 12.9 mg/dl within the first week of life formed the idiopathic hyperbilirubinemia (n: 50) and control (n: 54) groups, respectively. Thymine-adenine (TA) repeats in the promoter region of the UGT1A1 gene were investigated by polymerase chain reaction (PCR)-based non-radioactive DNA sequencing. In the idiopathic hyperbilirubinemia group, higher peak bilirubin levels, higher heterozygous and variant homozygous genotypes, higher TA7 allele frequencies, and shorter peak time were observed ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.05$, respectively). In conclusion, healthy breast-fed Turkish neonates who carry heterozygous and variant homozygous genotypes in the UGT1A1 gene are at high risk of developing significant hyperbilirubinemia without additional icterogenic factors.

Key words: hyperbilirubinemia, ABO incompatibility, promoter polymorphism, glucose-6-phosphate dehydrogenase deficiency, Gilbert syndrome, breast-feeding.

Jaundice is a significant neonatal problem, and pediatricians are anxious in encountering the potential neurotoxic effects of bilirubin. While most cases of newborn jaundice are physiologic, approximately 13.4% of the cases are nonphysiologic¹. In pathological jaundice, increased production of bilirubin, deficiency in hepatic uptake, impaired conjugation of bilirubin, and/or increased enterohepatic circulation of bilirubin are observed. However, there is no identifiable factor in almost half of the cases². Studies have shown that neonates of the African race have lower serum bilirubin levels, and Asian infants develop higher values when compared to their white counterparts³. While the incidence of severe hyperbilirubinemia is 4.6% in the United States⁴ and 5.1% in Israel⁵, it has been found to be 10.5% for healthy term newborns, and

25.3% for babies near to term in Turkey⁶. This observation supports the hypothesis that genetic risk factors may contribute to the development of pathological jaundice. The mutations in the promoter region and exon of the UDP-glucuronosyltransferase 1A1 (UGT1A1) enzyme gene that is responsible for bilirubin conjugation lead to structural and functional defects causing a 30-70% decrease in enzyme activity^{7,8}. It has been reported that heterozygous or homozygous mutation in exon 1 of the UGT1A1 gene (Gly71Arg) in Asians and homozygous promoter polymorphism coexistent with icterogenic factors such as ABO incompatibility, glucose-6-phosphate dehydrogenase (G6PD) deficiency, thalassemia or hereditary spherocytosis in whites are key factors for neonatal hyperbilirubinemia⁹⁻¹⁴.

In the present study, the association between

nonhemolytic unconjugated hyperbilirubinemia and promoter polymorphism of the UGT1A1 gene was investigated in healthy breast-fed Turkish neonates in the Denizli region who had significant hyperbilirubinemia with unexplained etiology or direct Coombs'-negative (DC)(-) ABO incompatibility.

Material and Methods

A total of 199 Turkish neonates with a gestational age of ≥ 38 weeks and with a birth weight of > 2500 g followed at the Pamukkale University Hospital and Denizli State Hospital were enrolled into the current investigation. Newborns with known risk factors including severe congenital malformation, infection, birth asphyxia, maternal diabetes, Rh, subgroup and DC(+) ABO incompatibility or hemolysis for any reason, liver disease, hypothyroidism, polycythemia, G6PD deficiency, cephalohematoma, dehydration, and insufficient feeding ($\geq 10\%$ loss of birth weight) were excluded from the study.

The control group consisted of 98 healthy newborns delivered in Pamukkale University Hospital whose peak serum total bilirubin (STB) levels were ≤ 12.9 mg/dl in the first week of life¹⁵⁻¹⁷. 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. In this group, complete blood count, peripheral blood smear, blood type, and DC and thyroid function tests were performed in addition to determination of STB concentration. Forty-four newborns of the control group were excluded from the study because of insufficient feeding⁸, prolonged jaundice (if visible jaundice exceeds 10 days) (16), and failure of polymerase chain reaction (PCR) (13) and DNA sequencing analysis⁷. Of the 54 newborns who formed the control group, 26 and 28 were ABO compatible and DC(-) ABO incompatible, respectively.

The idiopathic hyperbilirubinemia group contained 101 newborns who admitted to Pamukkale University Hospital and Denizli State Hospital with the diagnosis of hyperbilirubinemia (STB levels ≥ 17 mg/dl¹⁸). In this study group, serum direct and indirect bilirubin levels, reticulocyte count, G6PD, liver function tests, urine culture,

and if necessary C-reactive protein (CRP) determination were performed in addition to the parameters in the control group. Fifty-one newborns of the idiopathic hyperbilirubinemia group were excluded from the study because of insufficient feeding (7), Rh and ABO hemolytic diseases (4), elevated CRP and transaminases (3), cephalohematoma (3), urinary infection (2), polycythemia (2), hypothyroidism (2), G6PD deficiency (2), maternal diabetes (1), congenital malformation (1), or failure of PCR (15) or DNA sequencing analysis (9). Of the 50 newborns who formed the idiopathic hyperbilirubinemia group, 26 and 24 were ABO compatible and DC(-) ABO incompatible, respectively.

Serum total bilirubin (STB) levels were measured by spectrophotometric method (B-105 Digital bilirubinometer, Erma Inc, Japan). G6PD enzyme activities were also measured spectrophotometrically using a commercial kit (Trinity Biotech Procedure No: 345-UV, Ireland). Newborns with G6PD levels less than 4.6 U/g Hb were accepted as G6PD-deficient. DC test was performed by gel centrifugation, a sensitive technique for identifying IgG-coated red blood cells¹⁹. ABO incompatibility was defined if the mother's blood group was O, and her infant's blood type was A or B. The highest STB level measured in the first week of life was accepted as the peak bilirubin level. The period up to the peak bilirubin level was accepted as peak time. Because of the ongoing "baby-friendly hospital project" in Denizli, all of the babies included in the study were fed exclusively with breast-milk. Informed consents were obtained from the parents of all infants for the blood sampling and for the study. The study was approved by the Ethics Committee of Pamukkale University, Faculty of Medicine.

Sequence Analysis of UGT1A1: Blood samples were collected in EDTA vacutainers from all patients and controls. Genomic DNA was isolated by standard phenol-chloroform procedure. Oligonucleotide primers were used to amplify and sequence the fragment of 495 bp in size according to Huang et al.⁸ for the identification of thymine-adenine (TA) repeats. The amplification reaction mixture contained 1 μ l DNA in 4 deoxynucleotide triphosphates (5 μ l each, 10 pmol/ μ l) 3 μ l each primer- 5

μl MgCl_2 , (1 U/ μl) - 5 μl of Taq Polymerase, and 1x buffer. The PCR reaction was performed with a DNA thermal cycle as follows: 30 cycles at 94°C for 30 s, 55°C for 15 s, 72°C for 1 min, and 72°C for 3 min. Amplification of the PCR products was confirmed by 2% agarose gel electrophoresis. PCR products were treated with EZ10-Spin Column PCR Purification Kit (BIO BASIC Inc., Ontario, Canada) to be used as DNA sequencing templates. DNA sequencing was done by Dye Terminator Cycle Sequencing with Beckman CEQ 8000 Genetic Analysis System (Beckman Coulter Inc., Fullerton, CA, USA) according to the manufacturer's instructions.

Patients were classified according to the promoter sequence of the gene encoding UGT1A1 as normal homozygote, bearing the sequence (TA)6TAA in the TATAA element of the promoter of both alleles (TA6/6), variant homozygote with the sequence (TA)7TAA in both alleles (TA7/7, Gilbert), and heterozygote with one of each in the respective allele (TA6/7). The rate of individuals who carry UGT1A1 gene TA7 allele in a society was described as TA7 allele frequency.

Statistical Methods: The data are expressed as means \pm standard deviation (SD). Student t test was used to identify the statistical difference between the data of two groups, with respect to birth weight, gestational age, peak STB levels, and peak time. Chi-square test was used to compare the distribution of genotypes and A(TA₇)TAA allele frequencies in the two groups. Peak STB levels according to genotypes were tested with variance analysis (post-hoc Tukey, HSD). Statistical significance was set at $p < 0.05$.

Results

When the demographic characteristics were analyzed, no statistically significant difference was found in terms of gestational age and birth weight between the two groups ($p > 0.05$). The average peak STB levels were higher and the peak time of STB was significantly shorter in the idiopathic hyperbilirubinemia group than in the control group ($p < 0.001$ and $p < 0.05$, respectively) (Table I). Among the 104 infants studied in the current investigation, TA7/7, TA6/7, and TA6/6 genotypes were found at

rates of 5.8%, 37.5%, and 56.7%, respectively. TA6/7 and TA7/7 genotypes were frequently observed in the idiopathic hyperbilirubinemia group (68%, 12%) compared to the control group (11.2%, 0%) ($p < 0.001$) (Table II). TA7 allele frequency was higher in the idiopathic hyperbilirubinemia group (0.45) compared to the control group (0.05) ($p < 0.001$) (Table II). TA5 and TA8 polymorphisms were not found in this study.

According to TA6/6, TA6/7 and TA7/7 genotypes, peak STB levels of all 104 infants were found as 10.6 ± 3.8 , 18.3 ± 3.9 and 22.8 ± 2.2 , respectively. The increase in peak STB level was statistically significant in the idiopathic hyperbilirubinemia group ($p < 0.001$), whereas this increase was not significant in the control group ($p > 0.05$). In the idiopathic hyperbilirubinemia group, peak STB levels of newborns with TA6/7 and TA7/7 were higher than in those with TA6/6 ($p < 0.001$). Similarly, there was a significant difference in the peak STB levels between the newborns with TA6/7 and those with TA7/7 genotypes ($p < 0.001$) (Table III).

Discussion

In the present study, both TA7/7 and TA6/7 promoter polymorphisms in the UGT1A1 gene were found as risk factors in Turkish newborns with significant hyperbilirubinemia of unknown etiology or DC(-) ABO incompatibility. The frequency of TA7/7 (Gilbert) genotype was detected as 5.8% in our study, but it has previously been reported as 0.6%, 8.5%, 9%, and 10% in other studies from different regions of Turkey^{16,18,20,21}. It was already reported that coexistence of the TA7/7 promoter polymorphism with icterogenic factors such as ABO incompatibility, G6PD deficiency including female heterozygotes, thalassemia or hereditary spherocytosis causes severe indirect hyperbilirubinemia^{11-14,22}. In the absence of the additional icterogenic factors, the TA7/7 genotype may be associated with higher STB levels, but does not necessarily lead to significant hyperbilirubinemia. In the study of Kaplan and coworkers¹², while the UGT1A1 promoter polymorphism did not cause severe hyperbilirubinemia in newborns without G6PD deficiency, TA6/7 and TA7/7 promoter polymorphisms increased the risk of severe

Table I. Mean (\pm SD) Gestational Age, Birth Weight, Peak Serum Total Bilirubin (STB) Levels and Peak Duration According to Groups

	Idiopathic	
	Hyperbilirubinemia Group (n= 50)	Control Group (n= 54)
Gestational age (weeks)	38.8 \pm 0.6	38.8 \pm 0.7
Range	38-40	38-40
Birth weight (g)	3393 \pm 276	3391 \pm 274
Range	2800-3880	2650-3880
Peak STB levels (mg/dl)	19.7 \pm 1.6*	9.1 \pm 1.3
Range	17.0-25.0	5.3-11.5
Peak time (days)	3.6 \pm 0.8**	5.0 \pm 1.0
Range	3-5	3-7

Comparison of idiopathic hyperbilirubinemia group with control group in terms of peak STB levels *($p < 0.001$), and peak time **($p < 0.05$)

Table II. Distribution of Genotypes and A(TA₇)TAA Allele Frequencies

GROUPS	TA6/6	TA6/7	TA7/7	Allele Frequencies
	n (%)	n (%)	n (%)	n TA7
Idiopathic hyperbilirubinemia group (n: 50)	10 20*	34 68*	6 12*	50 0.45*
Control group (n: 54)	48 88.8	6 11.2	0 0	54 0.05

Comparison of idiopathic hyperbilirubinemia group with control group in terms of TA6/6, TA6/7, TA7/7 frequencies and TA7 allele frequencies *($p < 0.001$)

Table III. Peak Serum Total Bilirubin Levels of Groups According to Genotypes

GROUPS	Peak	Bilirubin	Levels (mg/dl)
	TA6/6	TA6/7	TA7/7
Idiopathic hyperbilirubinemia group (n: 50)	18.5 \pm 0.8	19.9 \pm 1.0*	22.8 \pm 2.2*
Control group (n: 54)	9.0 \pm 1.4	9.4 \pm 1.2	0

Comparison of peak bilirubin levels of TA7/7 and TA6/6, TA6/7 and TA6/6, TA7/7 and TA6/7 genotypes in idiopathic hyperbilirubinemia group *($p < 0.001$)

hyperbilirubinemia (STB >15 mg/dl) by a dose-dependent genetic interaction in newborns with G6PD deficiency. In Italian G6PD-deficient neonates, homozygosity for the variant TA7/7 promoter did not apparently increase the risk of hyperbilirubinemia²³. In the previous studies performed in the Turkish population, no relationship was detected between the promoter polymorphism on the UGT1A1 gene and idiopathic neonatal hyperbilirubinemia^{16,18,20,21}. The divergent results in these studies might be attributed to the ethnic and geographical characteristics and different criteria in patient selection. Laforgia and coworkers²⁴ found a significantly higher incidence of homozygosity (26.8%) for the variant TA7/7 promoter in neonates (excluding hemolytic conditions) with STB >13.0 mg/dl compared to those of control groups whose STB values did not exceed that concentration (12.2%). Similarly, in our study, both TA7/7 (12% vs 0%) and TA6/7 (68% vs 11.2%) promoter polymorphism frequencies were found higher in the idiopathic hyperbilirubinemia group than in those of the control group without icterogenic factors. The current demonstration of a reduction in hepatic tissue UGT enzyme activity, 37% in heterozygotes and 52% in homozygotes for the UGT promoter polymorphism, now provides a biochemical basis for the clinical manifestations observed in the neonates⁷.

In newborns with DC(-) ABO incompatibility, no increased hemolysis or hyperbilirubinemia is observed, and the clinical view is similar to that of the newborns with no ABO incompatibility²⁵. Herschel and associates¹⁹ suggested investigating increased bilirubin production without isoimmunization such as G6PD deficiency and erythrocyte membrane defects in newborns with severe hyperbilirubinemia and DC(-) ABO incompatibility. Kaplan et al.¹¹ reported that the presence of TA7/7 UGT1A1 promoter polymorphism increases the risk of hyperbilirubinemia (≥ 15 mg/dl) in newborns with DC(-) ABO incompatibility. In the present study, TA6/7 promoter polymorphism was found as a risk factor in addition to TA7/7 in newborns with DC(-) ABO incompatibility (Table II).

It was reported that TA7/7 promoter polymorphism of UGT1A1 caused accelerated increase in neonatal jaundice^{26,27}. The present

study found that the peak time of STB was shorter in the idiopathic hyperbilirubinemia group compared to the control group. The explanation is that phototherapy may have prevented further elevation in STB levels and may have shortened the peak time in the idiopathic hyperbilirubinemia group.

Raijmakers et al.⁷ demonstrated that not only homozygosity but also heterozygosity for the UGT1A1 promoter polymorphism results in a significant decrease in expression of enzymatic activity of hepatic UGT compared to the wild type. The increase in peak STB levels was positively correlated with the frequency of the TA7 allele in the idiopathic hyperbilirubinemia group. In newborns with TA7/7 genotype, the peak STB levels were higher than in those with TA6/7 and TA6/6 genotypes in the idiopathic hyperbilirubinemia group.

In contrast to the TA7/7 UGT1A1 promoter polymorphism, both hetero- and homozygosity for the G71R mutation and combination of the UGT1A1 promoter and coding region mutations were associated with hyperbilirubinemia without any additional icterogenic factors²⁸. In addition to the genetic abnormalities in bilirubin conjugation, mutations of the organic anion transporter 2 (OATP2) gene, which has a role in the hepatic uptake of bilirubin, could be effective in severe neonatal hyperbilirubinemia^{10,29}. While the clinical effect of TA6/7 heterozygosity alone is probably minimal, the combined effect of lactation failure during the initial stages of breastfeeding enhances heme oxygenase activity (i.e. increased bilirubin production), and enterohepatic bilirubin circulation (i.e. increased bilirubin load)²⁹. Although the mutations in the UGT1A1 exon and OATP2 gene were not investigated in the Turkish newborns, coexistence of exclusive breastfeeding and TA6/7 or TA7/7 promoter polymorphism might be suggested as a contributing factor for the development of severe hyperbilirubinemia by a dose-dependent genetic interaction.

In conclusion, among breast-milk-fed Turkish newborns, homozygous and heterozygous promoter polymorphisms of the UGT1A1 gene are risk factors for development of significant hyperbilirubinemia of unknown etiology or DC(-) ABO incompatibility. However, in the high-risk groups of hyperbilirubinemia,

combination of OATP2, UGT1A1 promoter and coding region mutations should be investigated further in Turkish newborns whose ancestors moved from Asia to Anatolia. Because of the discrepancies between the results of similar studies from our country, we suggest that the new studies should be undertaken with larger groups of infants on a nationwide basis.

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