Genotype Frequencies of UDP-Glucuronosyltransferase 1A1 Promoter Gene Polymorphism in the Population of Healthy Croatian Pre-Scholars

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ABSTRACT

Increased serum bilirubin levels in patients with Gilbert syndrome (GS) are caused by reduction of hepatic activity of bilirubin glucuronosyltranferase to about 30% of normal. UGT1A1 genetic polymorphism with absent or very low bilirubin UDP-glucuronosyltransferase (B-UGT) activity is associated with Gilbert's syndrome (GS) and other hyperbilirubinemias. The genetic basis of GS is the insertion of two additional TA nucleotides (resulting in seven repeats of TA) in the TATAA box, present in proximal promoter of UGT1A1 gene. This study included 323 Croatian pre-scholars, including 164 boys and 159 girls. Statistical analysis showed significant difference for total bilirubin concentration between different genotypes (p<0.001). Also, statistically significant difference for total bilirubin concentration was emphasized between genotypes 6/6 and 7/7 (p<0.001) as well as 6/7 and 7/7 (p<0.001). Higher total plasma bilirubin concentrations are significantly correlated with 7/7 genotype which is present in 9.8 % of population studied.

Key words: *unconjugated hyperbilirubinemia, Gilbert syndrome, UGT1A1 gene, polymorphism*

Introduction

Gilbert syndrome was described for the first time in 1901 by French physicians Gilbert and Lereboullet. It is a hereditary chronic disorder with 3–7% prevalence in general population. The main characteristics of the syndrome are mild unconjugated hiperbilirubinemia, absence of bilirubinuria, hemolysis or functional liver disorder[1].

Unconjugated bilirubin is a result of hem catabolism from hemoglobin and other hemoproteins. In hepatocytes the conjugation of nonpolar and compounds poorly soluble in water is a process of biotransformation important for detoxification and excretion of those compounds in bile. The UDP-glucuronosyltransferases (UGTs) are a family of enzymes responsible for their glucuronidation. Conjugation of bilirubin with glucuronide is catalysed by the hepatic enzyme bilirubin UDP-glucuronosyltransferase (B-UGT). The result of glucuronidation is the for-

mation of bilirubin mono- and di-glucuronide which are excreted into bile[1]. Increased serum bilirubin levels in patients with Gilbert syndrome (GS) are caused by reduction of hepatic activity of bilirubin glucuronosyltranferase to about 30% of normal[2,3].

UGT1 gene encodes 13 different UDP-glucuronosyltransferase genes. It is located on chromosome 2q37 and spreads 218 kb in length. It has a complex structure composed of 4 common exons (2–5) and 13 aminoterminal exons (1A1–1A13)[2,4]. Physiologically important isoform for bilirubin conjugation is coded by exon 1A1 and common exons 2–5[4].

UGT1A1 genetic polymorphisms without or very low B-UGT activity are associated with Gilbert's syndrome and other hyperbilirubinemias such as and Criggler-Najjar's syndrome type I and type II[5].

The genetic basis of GS is the insertion of two additional TA nucleotides in the TATAA box present in the proximal promoter part of UGT1A1 gene. As a result of the insertion UGT1A1*28 allele is formed. The transcription of the preformed gene is reduced to 20% and bilirubin glucuronidation decreased, in homozygous variants even by 80%[2]. However, smaller percentage of homozygous subjects has normal bilirubin serum concentration which indicates that mutation in the promoter region of this gene is not the only cause for manifestation of the syndrome[3]. Additional factors responsible for syndrome manifestation could be reduced lifetime of red blood cells or defects in hepatic transport mechanism causing decreased hepatic uptake of unjconjugated bilirubin[6.]

The aim of our study was to explore genotype frequencies of the UDP-glucuronosyltransferase 1A1 gene polymorphism in the TATAA box in the population of Croatian pre-scholars in order to define early risk for GS and other hyperbilirubinemias. We also investigated the association between different genotypes and plasma bilirubin concentrations.

Materials and Methods

Study subjects

Study subjects were recruited among Croatian pre--scholars in the city of Pula, who underwent routine medical examination for primary school registration. The study included 323 pre-scholars with of median age 6 ± 0.5 (155 girls and 164 boys). The blood samples were collected in June 2006 during the routine medical examinations for primary school registration at the division for school medicine in Pula General Hospital. Whole blood was collected into EDTA Vacutainer tubes (Terumo Europe, Belgium) for DNA extraction and plasma separation. Considering the fact that examinees were under age, parents were asked to give an informed consent for children to participate in the study. Study was approved by the Ethical Committee of Pula General Hospital and Medical School University of Zagreb.

Determination of plasma bilirubin concentration

Total plasma bilirubin concentration was determined by standard photometric method with diazo reagents on a Hitachi 717 (Roche, Switzerland). Samples with conjugated bilirubin measured values higher than 6 µmol/L were excluded from statistical analysis.

DNA analysis

DNA was isolated by the salting out method[7]. In order to analyze TA insertion in the promoter region of the UGT1A1 (TATAA box) amplification product was generated by polymerase chain reaction (PCR) using primers to amplify 253–255 bp long fragment, as described previously[8]. PCR was performed in a total volume of 25 µL containing 8 ng of template DNA, 0.2 mM of deoxynucleotides, 1.5 mM buffer with $MgCl_2$, 0.2 mM of each

primer and 0.75 units of Fast start Taq polymerase (Roche Diagnostics, Switzerland). Amplification was performed using GeneAmp PCR System 2700 (Applied Biosystems, USA). The PCR conditions included 25 cycles with the temperature profiles as follows: denaturation at 95° for 30 seconds, annealing at 62° for 40 seconds and extension at 72° for 40 seconds between initial denaturation at 95° for 5 minutes and final extension at 72° for 5 minutes.

The amplification was confirmed by 1.5% agarose gel electrophoresis. Amplified fragments were resolved on Spreadex EL 500 gels (Elchrom Scientific, Switzerland) using Elchrom Submerged Gel Electrophoresis System (Elchrom Scientific, Switzerland). Gels were run at 144 Volts for 4 h, than stained with SybrGreen I (Roche Diagnostics, Switzerland) for 45 min. and destained with Destaining solution (Elchrom Scientific, Switzerland) for 20 min. Genotypes were assigned as follows: homozygous for A(TA) $_6$TAA allele (6/6), heterozygous with one A(TA)$_6$TAA and one A(TA)$_7$TAA allele (6/7) and homozygous for A(TA)$_7$TAA allele (7/7) (Figure 1).

Statistical analysis

Statistical analysis was performed using a StatSoft, Inc. (1984–2005) STATISTICA version 7.1. Genotype frequencies were calculated by counting. Chi-square test was used for determination of Hardy-Weinberg equilibrium. Bilirubin concentrations between different genotypes were compared using nonparametric Kruskal--Wallis and Mann-Whitney test. Multivariate analysis (MANOVA) with gender as covariate was used for testing the effect of UGT1A1 promoter polymorphism on total
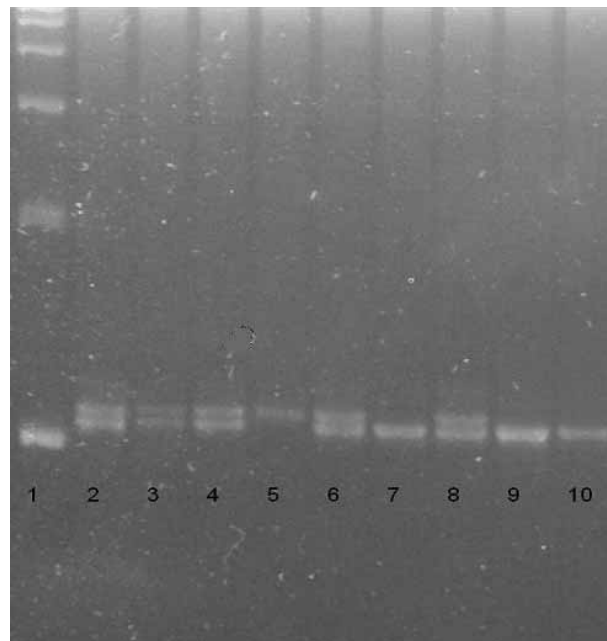

Fig. 1. Electrophoresis of the PCR-amplified fragments, 253–255 bp long in Spreadex EL 500 (1–50 bp marker; 2, 3, 4, 6, 8-genotype 6/7; 5-genotype 7/7; 7, 9, 10-genotype 6/6).

TABLE 1.
TOTAL BILIRUBIN CONCENTRATION IN DIFFERENT UGT1A1 GENOTYPES

Genotype	N	Total bilirubin concentration μmol/L			
		Median	Range	Kruskal-Wallis	MANOVA
6/6	129	6.2	3.1–16.6		
6/7	161	6.4	3.1–23.7	p<0.001	p<0.001
7/7	33	9.02	2.7–24.7		

bilirubin concentration. Total bilirubin concentrations were log-transformed for testing with MANOVA.

Results

In 323 samples the frequencies of genotypes 6/6, 6/7 and 7/7 genotype were 38.4%, 47.9% and 9.8%, respectively. Genotype frequencies were in Hardy-Weinberg equilibrium. Total bilirubin concentrations in all three genotypes are reported in Table 1 and Figure 2. Bilirubin concentration did not show normal distribution (test of homogeneity of variances, p<0.001). Statistical analysis showed significant difference for total bilirubin concentration between different genotypes (p<0.001). Also, statistically significant difference for total bilirubin concentration was emphasized between genotypes 6/6 and 7/7 (p<0.001) as well as 6/7 and 7/7 (p<0.001). Multivariate analysis showed that there were no changes in statistical significance after corrections by gender.

Out of 323 subjects only 6 had total bilirubin concentration higher than the upper limit of the plasma total bilirubin reference interval (≥19 mmol/L). Five of them were genotyped as 7/7 with median plasma bilirubin concentrations 23.9 (minimum: 21.3; maximum: 34.3) and one genotyped as 6/7.

Discussion

The result of our study showed significant correlation between total plasma bilirubin concentrations and 7/7
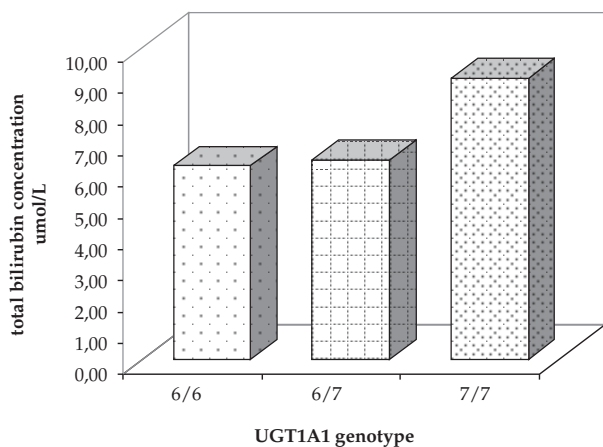

Fig. 2. Total bilirubin concentration in different UGT1A1 genotypes.

genotype. The frequency of 7/7 genotype was 9.8%. Similar results were reported elsewhere[2,8–11].

Significantly higher concentrations of total bilirubin were found in 7/7 genotype when compared to 6/6 and 6/7 genotypes. Even though some studies reported that significant difference exist between the 6/7 and 6/6 genotypes[3] this was not the case in our study.

Moreover, only 6 subjects had elevated total plasma bilirubin concentrations over the upper limit of the bilirubin reference values which indicates that additional factors could have an important role for the syndrome manifestation. One out of those six subjects had genotype 6/7 which indicated that heterozygous form of the UGT1A1 promoter genotype could also be associated with GS.

The frequency of the UGT1A1*28 allele and 7/7 genotype is different among ethnic groups. Burchell and Hume[5] summarized the data of several studies showing that the frequency of 7/7 genotype varies in different populations as follows: Caucasians up to 11%, in African population up to 23% and in Asians less than 3%[5]. Ethnic groups did not differ only in 7/7 genotype frequencies but also in total serum bilirubin concentrations. Beutler et al.[12] reported an inverse relation between UGT1A1 promoter genotype and bilirubin concentration in ethnic groups. Even though the frequency of 7/7 genotype was very high in African population the average bilirubin concentration was lower than predicted. According to this finding it is likely that there are other genetic differences except UGT1A1 promoter polymorphism in bilirubin metabolism between Africans and Caucasians. Furthermore, numerous mutations of UGT1A1 gene were found in Japanese population but the prevalence of UGT1A1*28 allele was much lower than in the Caucasian population[13]. Taken together, all this indicates that there are differences in TATAA box mutations as well as difference in genetic basis of GS between different ethnic groups.

As previously mentioned, the insertion of two additional TA nucleotides (resulting in seven repeats of TA) in the TATAA box in both alleles of UGT1A1 gene indicate a close association with reduced expression of the enzyme B-UGT. Raijmakers et al.[9] showed that B-UGT enzyme activity was significantly higher in 6/6 than in 6/7 and 7/7 genotype. They also noticed that in 6/7 genotype enzyme activity was significantly lower than in 6/6 genotype indicating that GS can be associated also with 6/7 genotype.

Glucuronidation is an important step in the process of detoxification, in which the toxic compounds are conjugated and eliminated through the bile. UGT1A1 genetic polymorphism, due to reduced activity of B-UGT enzyme, causes reduced glucuronidation of some drugs. Several investigators reported decreased glucuronidation and elimination of irrinotecan, an anticancer drug, in GS patients[14]. Active metabolite of irrinotecan SN-38 is undergoing glucuronidation by B-UGT and forming the inactive metabolite (SN-38-glucuronide), which is excreted into the bile[15]. Due to reduced activity of B-UGT, subjects with GS submitted to anticancer treatment could be at increased risk of irrinotecan toxicity[15]. Since UGT1A1 is responsible for conjugation of some other drugs and xenobiotics GS subjects could have increased susceptibility toward those compounds and be at increased risk of toxicity. For example, UGT1A1 was recognized to be one of several UGTs which glucuronidate carcinogens including benzo(a)pyrene (polycyclic aromatic hydrocarbon)[16]. Thus, subjects with reduced UGT1A1 activity could be at increased risk for developing cancer.

In their study Peterson et al.[17] indicated that UGT1A1 activity could be modulated by diet. They reported that subjects with 7/7 genotype have reduced bilirubin concentrations with increased intake of cruciferous vegetables suggesting that some components of those vegetables could influence UGT1A1 expression through signaling pathways.

In conclusion, the prevalence of genotype 7/7 in the population of Croatian pre-scholars is 9.8 % which correlates with the data of some similar Caucasians studies. Advanced genomic knowledge and technologies as well as the genetic background of common diseases and syndromes (like those involved in bilirubin metabolism) are starting to reveal great variety of potential genetic causes with important influence of environmental factors[18].

Considering the fact that UGT1A1*28 is a frequently occurring mutation in population, it would be suggested to introduce UGT1A1 genotyping in cases of borderline hyperbilirubinemia. Moreover, it would help us to distinguish whether elevated bilirubin concentrations in some subjects are the result of UGT1A1 polymorphism or other disorder of liver metabolism. Furthermore, it would enable more appropriate dosage adjustment of therapeutic drugs which are metabolized through UGT1A1, in GS patients.

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FREKVENCIJA GENOTIPOVA UDP-GLUKURONOZILTRANSFERAZE 1A1 POLIMORFIZMA U PROMOTOR REGIJI GENA U POPULACIJI ZDRAVE DJECE PREDŠKOLSKE DOBI U HRVATSKOJ

SAŽETAK

Povišena koncentracija bilirubina u pacijenata s Gilbertovim sindromom (GS) uzrokovana je redukcijom aktivnosti jetrene bilirubin glukuronoziltransferaze na otprilike 30%. Polimorfizam gena za UGT1A1 s dokinutom ili jako smanjenom aktivnošću enzima UDP-glukuronoziltransferaze (B-UGT) je povezan s Gilbertovim sindromom te drugim vrstama hiperbilirubinemije. Genetska osnova GS je insercija dva dodatna TA nukleotida (što rezultira sedmerostrukim ponavljanjem TA) u TATA kutiji prisutnoj u proksimalnoj regiji promotora gena za UGT1A1. U ovoj studiji sudjelovalo je 323 hrvatske djece predškolske uzrasti uključujući 164 dječaka i 159 djevojčica. Statistička analiza pokazala je značaj-

nu razliku u koncentraciji ukupnog bilirubina između genotipova (p<0.001). Nadalje, utvrđena je statistički značajna razlika u koncentraciji ukupnog bilirubina između genotipova 6/6 i 7/7 (p<0.001), kao i 6/7 i 7/7 (p<0.001). Povišene koncentracije ukupnog bilirubina u plazmi pokazale su značajnu povezanost sa genotipom 7/7 koji je bio prisutan u 9.8% populacije.