UGT1A1 $(TA)_n$ promoter genotype: diagnostic and population pharmacogenetic marker in Serbia

Running title: *UGT1A1* genotyping in Serbia

Marija Vukovic[1], Nedeljko Radlovic[2], Zoran Lekovic[2], Ksenija Vucicevic[3], Nina Maric[4], Nikola Kotur[5], Vladimir Gasic[5], Milena Ugrin[5], Maja Stojiljkovic[5], Lidija Dokmanovic[6], Branka Zukic[5], Sonja Pavlovic[5*]

[1] Department for Medical Genetics, University Clinical Centre of the Republic of Srpska, Banja Luka, Bosnia and Hercegovina

[2] Department of Gastroenterology and Nutrition, University Children's Hospital, School of Medicine, University of Belgrade, Belgrade, Serbia

[3] Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

[4] Clinic for Children Diseases, University Clinical Centre of the Republic of Srpska, Banja Luka, Bosnia and Hercegovina

[5] Laboratory for Molecular Biomedicine, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia

[6] Department of Hematology and Oncology, University Children's Hospital, School of Medicine, University of Belgrade, Belgrade, Serbia

*Corresponding author:

Sonja Pavlovic
Laboratory for Molecular Biomedicine
Institute of Molecular Genetics and Genetic Engineering, University of Belgrade
Vojvode Stepe 444a, PO BOX 23
11010 Belgrade, Serbia
E-mail: sonya@sezampro.rs
Tel: (+) 38111 3976445
Fax: (+) 38111 3975808

Abstract

UGT1A1 enzyme is involved in metabolism of bilirubin and numerous medications. Non-conjugated hyperbilirubinemia, commonly presented as Gilbert syndrome (GS), is a result of decreased activity of UGT1A1 enzyme, Variable number of TA repeats in the promoter of the *UGT1A1* gene affects enzyme activity. Seven and 8 TA repeats cause a decrease of UGT1A1 activity -risk GS alleles, while 6 TA repeats contribute to normal UGT1A1 activity- non-risk GS allele. Also, the *UGT1A1* $(TA)_n$ promoter genotype is recognized as clinically relevant pharmacogenetic marker.

The aim of the study was to assess diagnostic value of *UGT1A1* $(TA)_n$ promoter genotyping in pediatric GS patients. Correlation of *UGT1A1* $(TA)_n$ genotypes and level of non-conjugated bilirubin at diagnosis and after hypocaloric and phenobarbitone tests in these patients was analyzed. Another aim of the study was to assess pharmacogenetic potential of *UGT1A1* $(TA)_n$ variants in Serbia.

Fifty-one pediatric GS patients and 100 healthy individuals were genotyped using different methodology - PCR followed by acrylamide electrophoresis, fragment length analysis and/or DNA sequencing.

Concordance of the *UGT1A1* $(TA)_n$ promoter risk GS genotypes with GS was found in 80% of patients. Therefore, *UGT1A1* $(TA)_n$ promoter genotyping is not reliable genetic test for GS, but it is useful for differential diagnosis of diseases associated with hyperbilirubinemia. Level of bilirubin in pediatric GS patients at diagnosis was *UGT1A1* $(TA)_n$ promoter genotype-dependent. We found that the frequency of pharmacogenetic relevant *UGT1A1* $(TA)_n$ promoter genotypes is 63%, pointing out that *UGT1A1* $(TA)_n$ promoter genotyping could be recommended for preemptive pharmacogenetic testing in Serbia.

Key words: Gilbert syndrome, non-conjugated hyperbilirubinemia, population pharmacogenetics, *UGT1A1* $(TA)_n$ promoter variants

Introduction

Uridin diphospho-glucuronosyl transferases (UGT) are family of enzymes responsible for glucuronidation of numerous endobiotics, xenobiotics and drugs. Glucuronidation is a process of biotransformation of substrate to water soluble, mainly non-toxic products ready for excretion (1). Human UGT superfamily is divided into four families: UGT1A, UGT2, UGT3 and UGT8 (2). UGT1A family is encoded by *UGT1A* gene complex located on chromosome 2q37. This gene complex at 5' region has thirteen variable exons, linked to 4 common exons at 3' region of *UGT1A* gene. Each of thirteen 5' exons has its own TATA promoter elements. Four of 13 first exons are pseudoexons, and 9 exons are viable, independently transcribed as the first exon. Those 9 different first exons with highly conserved region from exon 2 to exon 5, generate 9 different transcripts (UGT1A1, UGT1A3 through UGT1A10). Each transcript has identical 3' ends and unique 5' end. Variable first exon provides substrate specificity, while highly conserved common exons contain site for interactions with UDP glucuronic acid as common substrate (3). *UGT1A1* gene is expressed in the liver, colon, intestine and stomach (4,5). UGT1A1 enzyme has main role in glucuronidation of bilirubin. There is no alternative metabolic pathways for efficiently detoxification and elimination of bilirubin. Currently, more than 130 *UGT1A1* variants have been reported as a cause of Gilbert syndrome, Crigler-Najjar syndrome type 1 and Crigler-Najjar syndrome type 2 (6). Allelic variations were found in both the exonic and promoter sequences (7). The most common variants detected in *UGT1A1* gene are different number of TA repeats in its promoter, rs8175347 (8,9) . Wild type *UGT1A1* contains six TA repeats [A(TA)6TAA] in its promoter region. *UGT1A1**28, *UGT1A1**37 and *UGT1A1**36 variants have seven, eight and five TA repeats respectively. Transcriptional activity of *UGT1A1* gene and, consequently, the activity of UGT1A1 enzyme, depends on number of TA repeats. Higher number of TA repeats causes decreased enzyme activity. Heterozygous status of *UGT1A1**28 results in 25% decreased enzyme activity and homozygous status of this variant reduced transcription activity by 70% (7). This leads to Gilbert syndrome (GS), a mild form of intermittent non-conjugated hyperbilirubinemia, that lacks hemolysis or hepatocellular injury. Therefore, 6 TA repeats in *UGT1A1* promoter is considered as a non-risk GS allele and 7 or 8 TA repeats are responsible for development of GS (risk GS allele).

GS is a hereditary benign condition, commonly presented in 3-13% in general population. GS is inherited in autosomal-recessive manner, but several cases have been reported as dominantly

inherited [10, 11]. GS is rarely clinically manifested until before puberty. Males are more often diagnosed than females, two to seven times, because there is a bigger bilirubin load per kilogram body weight in males, and/or androgen steroid hormone suppresses hepatic bilirubin clearance [12,13].

*UGT1A1*28* and *UGT1A1*37* variants have been shown to contribute to hyperbilirubinemia and increased likelihood of gallstone formation in several inherited hemolytic conditions [14]. They are also associated with increased risk of breast cancer, neonatal hyperbilirubinemia and the risk of developing gallstones in cystic fibrosis. Shorter erythrocyte life and damage in transport of non-conjugated bilirubin are further contributing to development of GS.

GS does not require therapy as it is considered to be a benign condition. However, fasting, excessive physical stress, febrile conditions and hormone changes during menstrual cycles additionally potentiate hyperbilirubinemia and could be treated with phenobarbitone which stimulates the UGT1A1 enzyme activity [15]. Sometimes, for cosmetically purposes GS could be treated to moderate the subicterus.

The complete or nearly complete lack of ability of glucuronidation of bilirubin is not compatible with life. Genetic variations within exons 2 to 5 of *UGT1A1* gene locus, resulting in total absence of bilirubin glucuronide formation, cause Crigler-Najjar syndrome type 1 (8). Also, some genetic variations in introns resulting in frameshifts and premature stop codon have been described. Lack of UGT1A1 activity leads to high level of bilirubin shortly after birth (20-50mg/dL), causing bilirubin encephalopathy, kernicterus and death. Crigler-Najjar syndrome type 1 could be managed by repeatedly exchange transfusions, long-term phototherapy, plasmapheresis, hemoperfusion and finally the only successful therapy is liver transplantation. Several single nucleotide variations in *UGT1A1* gene have been reported to cause substitution of single amino acid and reduce enzyme activity to less than 10% of normal activity, but total bilirubin levels do not exceed 20mg/dL. This condition is recognized as a Crigler-Najjar syndrome type 2 (15). In this case, optimal therapy is phenobarbital which stimulates UGT1A1 activity.

Taking into account that a number of medications are metabolized by UGT1A1 enzyme, *UGT1A1* $(TA)_n$ promoter variants are recognized as clinically relevant pharmacogenetic markers (16).

Presence of 7 or 8 TA repeats in promoter of *UGT1A1* gene could severely impact the ability to metabolize certain medications, including antineoplastic drugs used in oncology: irinotecan (solid tumors, lymphoma and colorectal cancer treatment), belinostat (peripheral T-cell lymphoma treatment), epirubicin (breast cancer treatment), 5-fluorouracil (colorectal cancer treatment), axitinib and busulfan (various hematological and non-hematological cancers), atazanavir and ritonavir (HIV infections treatment) and widely used analgesic/antipyretic drug acetaminophen (paracetamol). Alterations in hepatic metabolism may result in life-threatening toxicities in various organ systems [17,18]. Recommendations for pharmacogenomic testing for several of these drugs are given and has been already applied in routine clinical practice [19].

The aim of this study was to analyze if *UGT1A1* $(TA)_n$ promoter variants can be efficient diagnostic tool for easier and faster diagnosis of Gilbert syndrome, by using accurate and cost-effective methodology for detection of number of TA repeats in *UGT1A1* promoter. Correlation of *UGT1A1* $(TA)_n$ promoter genotypes, risk GS and non-risk GS, with levels of non-conjugated bilirubin at diagnosis and after hypocaloric diet and phenobarbitone tests in pediatric GS patients will be used for assessment of diagnostic value of *UGT1A1* $(TA)_n$ promoter variants genotyping.

Additionally, given into account that UGT1A1 enzyme is very important for metabolism of various drugs and that, in the case of presence of defined *UGT1A1* $(TA)_n$ promoter variants, numerous drugs can present unwanted side effects or toxicities, the aim of this study was to assess pharmacogenetic potential of those variants in Serbia. The results will point out if pharmacogenetic testing should be performed in Serbia before administration of medications whose metabolism is *UGT1A1* $(TA)_n$ genotype-dependent.

Materials and methods

Subjects

For this study, 51 blood samples were obtained from children who were previously diagnosed as positive for GS at University Children's Hospital, Belgrade, Serbia. Ethical approval was obtained from the Ethics committee of University Children's Hospital, University of Belgrade. Study was

conducted in accordance with the Declaration of Helsinki. Written informed consent for the molecular analyses was obtained from the participants' parent or guardian before the collection of the specimens. A 5ml Na-citrate tube of whole blood was obtained for each subject during routine clinical check-ups. All personal identifiers were removed; isolated DNA samples were tested anonymously.

Also, 100 unrelated healthy individuals (67 children and 32 adults) were enrolled in the study. Ethical approval was obtained from the Ethics committee of the Institute of Molecular Genetics and Genetics Engineering, University of Belgrade. Study was conducted in accordance with the Declaration of Helsinki. Written informed consent for the molecular analyses was obtained from all subjects before the collection of the specimens. In case of minor participants, written informed consent was obtained from the participants' parent or guardian and blood sample was taken during routine pediatric check-ups. A 5ml Na-citrate tube of whole blood was obtained for each subject. All personal identifiers were removed; isolated DNA samples were tested anonymously. All subjects enrolled in the study were unrelated healthy donors and self-declared as Serbs.

Hypocaloric diet and phenobarbitone testing

Levels of conjugated and total bilirubin was measured in GS patients group at diagnosis and after a three-day hypocaloric diet test (400-kcal per day) during standard laboratory examination. Further, a three-day phenobarbitone test (2 mg/kg/day) was performed in patients group and levels of conjugated and total bilirubin was measured also. Level of non-conjugated bilirubin was calculated as a mathematical difference between the total and conjugated levels of bilirubin.

***UGT1A1* genotyping**

***UGT1A1* $(TA)_n$ promoter genotyping**

DNA was extracted from blood samples using the QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). Number of TA repeats in *UGT1A1* promoter was genotyped as previously described [20] with slight modifications. The amplification reaction was performed in a total volume of 25 μl, and the reaction mix contained 20 pmol of each primer, 50-100 ng of genomic DNA, 200 μmol/l of each dNTP (Fermentas, ON, Canada), 1x PCR reaction buffer (Qiagen), 1x

Q solution (Qiagen), 2.75 mM MgCl2, 1 U HotStar DNA polymerase (Qiagen). The temperature profile of the PCR reactions was for the initial activation of DNA polymerase set at 95°C for 15 min, followed by 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 63°C, and 30 sec elongation at 72°C, ended by a final extension period of 7 min at 72°C. PCR fragments were visualized on 2% agarose gel and finally analyzed using 15% acrylamide electrophoresis (19:1 acrylamide/bisacrylamide in 1xTBE buffer, run at 300V and 60mA for 5h) stained with Ag-nitrate [21]. Each acrylamide electrophoresis run had a positive (TA 7/7) and negative (TA 6/6) control sample, previously confirmed using Sanger sequencing methodology.

Twenty percent of samples were randomly chosen and results of *UGT1A1* promoter genotyping by the PCR/acrylamide electrophoresis methodology were checked and confirmed using fragment length analysis of fluorescent PCR products [22]. The 12 μl PCR reaction contained 50ng of DNA, 1xPCR buffer (Qiagen), 15.4mM MgCl2, 0.4mM dNTPs, 1U of HotStar Polymerase (Qiagen) and 0.4μM of both UGT1A1F (FAM dye-labeled, 5'-famTACAGTCACGTGACACAG-3') and UGT1A1R (5'-TTTGCTCCTGCCAGAGGTTCG-3') primers. The temperature profile of the PCR reactions was for the initial activation of DNA polymerase set at 95°C for 15 min, followed by 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 55°C, and 30 sec elongation at 72°C, ended by a final extension period of 7 min at 72°C. PCR products were separated on an Applied Biosystems 3130 DNA Analyzer and the collected data were analyzed with the GeneMapper v4 software.

Ten percent of samples were randomly chosen and results of *UGT1A1* $(TA)_n$ promoter genotyping by the PCR/acrylamide electrophoresis methodology were checked and confirmed using Sanger sequencing methodology. Those samples were used as positive/negative controls on each acrylamide electrophoresis run [23].

UGT1A1 gene analysis

According to ENST00000305208.9, primers were designed to analyze all 5 coding exons and nearby intronic sequences of *UGT1A1* gene. The amplification reaction was performed in a total volume of 30 μl, and the reaction mix contained 10 pmol of each primer, 50-100 ng of genomic DNA, 0.5 mM of each dNTP (Fermentas, ON, Canada), 1x PCR reaction buffer, 1.4 mM $MgCl_2$,

1 U DNA polymerase (KAPA Biosystems, USA). The temperature profile of the PCR reactions was for the initial activation of DNA polymerase set at 95°C for 15 min, followed by 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing as given in Table 1, and 30 sec elongation at 72°C, ended by a final extension period of 10 min at 72°C. PCR fragments were visualized on 2% agarose gel, separated on an Applied Biosystems 3130 DNA Analyzer and the collected data were analyzed with the GeneMapper v4 software.

Table 1. List of primers used for sequencing of the *UGT1A1* coding and nearby intronic regions.

Primer	Sequence	Annealing temperature (°C)
UGT1A1_ex1-1_F	5'- GCTACCTTTGTGGACTGACAGC-3'	60
UGT1A1_ex1-1_R	5'- CCATGAGCTCCTTGTTGTGCAG -3'	
UGT1A1_ex1-2_F	5'- GCCATTCCAAAGGGAGGATG-3'	57
UGT1A1_ex1-2_R	5'- GATGATGCCAAAGACAGACTCAAAC -3'	
UGT1A1_int1ex2_F	5'- CTGTAAGCAGGAACCCTTCCTC -3'	58
UGT1A1_int1ex2_R	5'- GGATTAATAGTTGGGAAGTGGCAGG -3'	
UGT1A1_ex3_F	5'- AAGTTGCCAGTCCTCAGAAGC -3'	60
UGT1A1_ex3_F	5'- TGTTACTCACATGCCCTTGCAG -3'	
UGT1A1_ex4int4_F	5'- TGCAAGGGCATGTGAGTAACAC-3'	58
UGT1A1_ex4int4_R	5'- GCACTCCAGCCTAGGTGAC -3'	
UGT1A1_ex5_F	5'- CAGGTTTCCTTTCCCAAGTTTGG -3'	58
UGT1A1_ex5_R	5'- CACTCTGGGGCTGATTAATTTATGC -3'	

Statistical analysis

All statistical analysis was performed using IBM's SPSS v.21 s software. The difference in *UGT1A1* $(TA)_n$ promoter genotype frequencies (risk and non-risk GS genotypes) between control group and GS patients was assessed using Fisher's exact test. *UGT1A1* $(TA)_n$ promoter genotype frequencies of control group were checked for Hardy–Weinberg equilibrium by exact test [24]. The distribution of non-conjugated bilirubin in GS patients was checked for normality using Shapiro-Wilk and Kolmogorov-Smirnov tests. The difference in the level of non-conjugated bilirubin between carriers of risk and non-risk GS genotypes was assessed using Mann-Whitney test. Probability values less than 0.05 were considered statistically significant. All tests were non-directional (two-tailed).

Results

UGT1A1 $(TA)_n$ promoter genotyping as a diagnostic tool for GS

Patients GS group consisted of 32 males (62.75%) and 19 females (37.25%) and median age was 16 (range 3 to 19 years).

The *UGT1A1* $(TA)_n$ promoter genotypes detected in the control and GS patients' groups were TA 6/6, TA 6/7, TA 7/7 and TA 7/8. The same *UGT1A1* $(TA)_n$ promoter genotypes were identified using PCR amplification followed by acrylamide electrophoresis stained with Ag-nitrate and/or using fragment length analysis methodology. In order to validate both methods, we used reference samples previously determined by DNA sequencing.

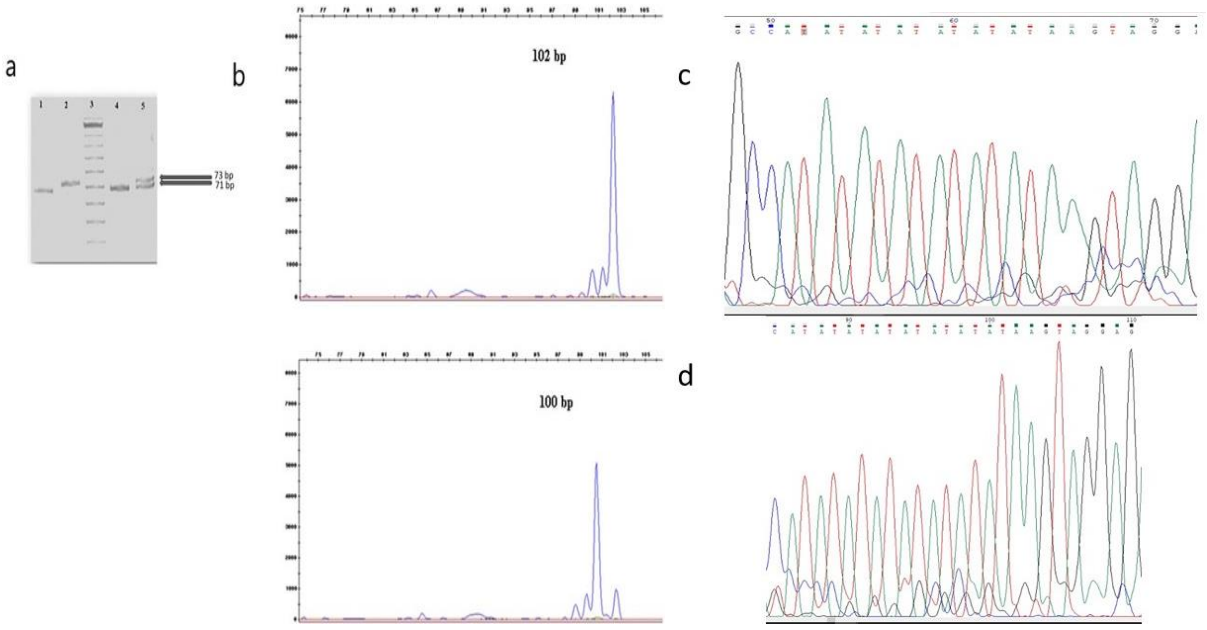

Figure 1.

a. *UGT1A1* $(TA)_n$ promoter PCR products on 15% acrylamide gel electrophoresis stained with Ag-nitrate. Wells 1 and 4 - 6/6 TA repeats (71bp), well 2 - 7/7 TA repeats (73bp), well 5 - 6/7 TA repeats (71bp and 73bp). Well 3 - 5bp DNA ladder.
b. Electophoretograms obtained with fragment length analysis of *UGT1A1* $(TA)_n$ promoter repeats. The upper one peak is 7/7 TA repeats (102bps). The lower one peak is 6/6 TA repeats (100bps).
c. Electophoretogram obtained with sequencing analysis of promoter of *UGT1A1* gene with 6/6 TA repeats.
d. Electophoretogram obtained with sequencing analysis of promoter of *UGT1A1* gene with 7/7 TA repeats.

The detection of the number of TA repeats in *UGT1A1* promoter using PCR amplification followed by 15% acrylamide electrophoresis stained with Ag-nitrate (Figure 1. a) and fragment analysis (Figure 1. b) are presented in Figure 1. Also, sequence of a TA 6/6 and TA 7/7 promoter repeats, used as controls in electrophoresis runs, are presented in Figure 1 (Figure 1 c. TA 6/6; and Figure 1 d. TA 7/7).

The distribution of *UGT1A1* $(TA)_n$ promoter genotypes in pediatric GS patients are given in Table 2. The *UGT1A1* $(TA)_n$ promoter genotype distribution in pediatric GS patients was as follows: TA 6/6 (3.92%), TA 6/7 (15.87%), TA 7/7 (76.47%) and TA 7/8 (3.92%).

Table 2. The distribution of *UGT1A1* $(TA)_n$ promoter repeats in GS patients and control groups

GS patients (n=51)			controls (n=100)		
	n	%		n	%
6/6	2	3.92	6/6	37	37
6/7	8	15.87	6/7	47	47
7/7	39	76.47	7/7	16	16
7/8	2	3.92	7/8	0	0

Due to small number of carriers of 6/6 and 7/8 *UGT1A1* TA promoter genotypes, for further analysis groups of 6/6 TA *UGT1A1* promoter genotypes and 6/7 TA *UGT1A1* promoter genotypes were considered as one, non-risk GS genotypes, as well as groups of 7/7 TA and 7/8 TA *UGT1A1* promoter genotypes, risk GS genotypes.

Carriers of risk GS genotypes have more than 21-fold higher odds for developing Gilbert syndrome than carriers of non-risk GS genotypes OR=21.5 [9.0 – 51.6], $p<10^{-14}$ (Fisher's exact test), pointing out the importance of molecular genetic testing in GS diagnostic pipeline.

The levels of total and direct bilirubin were measured at diagnosis and also after hypocaloric and phenobarbitone tests in pediatric GS patients (Table 3). The total and direct bilirubin levels did not follow normal distribution (Smirnof-Kolmogorov and Shapiro-Wilk tests, $p<0.05$).

Table 3. Levels of non-conjugated bilirubin in pediatric GS patients

	levels of non-conjugated bilirubin in GS risk group in μmol/l (average±st.dev.)	levels of non-conjugated bilirubin in GS non-risk group in μmol/l (average±st.dev.)	p values (Mann-Whitney test)
at diagnosis	27.51±19.16	16.92±7.57	0.079
after hypocaloric diet test	62.02±43.85	41.40±14.67	0.24
after phenobarbitone test	18.01±14.57	14.23±6.71	0.626

The difference between levels of non-conjugated bilirubin at diagnosis in pediatric GS patients were statistically significant between the non-risk GS genotype carriers and risk GS genotype carriers (Mann-Whitney test, p=0.079). After hypocaloric diet, the mean level of non-conjugated bilirubin was increased 2.91-fold in GS risk and 2.52-fold in GS non-risk genotype carriers in pediatric GS patients. This increase was not *UGT1A1* $(TA)_n$ promoter genotype-related (Mann-Whitney test, p=0.409).

After a three-day phenobarbitone test, levels of total and conjugated bilirubin were also measured in pediatric GS patients. The non-conjugated serum bilirubin fracture decreased 27.4% in GS risk and 6.4% in GS non-risk genotype carriers. The difference of a decrease of non-conjugated bilirubin levels after phenobarbitone test was statistically significant when the pediatric GS patients carrying GS risk were compared to GS non-risk genotypes (Mann-Whitney test, p=0.040) (Figure 2).

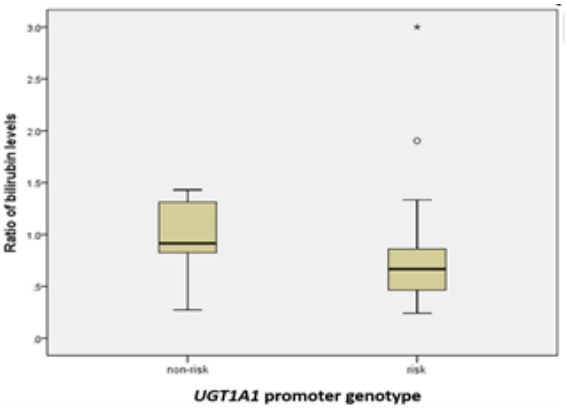

Figure 2. Median and interquartile range of ratio between levels of non-conjugated bilirubin after phenobarbitone test comparing to levels of bilirubin before the test non-conjugated bilirubin according to GS risk groups (GS non-risk, GS risk) (Mann-Whitney test, p=0.040)

UGT1A1 TA 7/7 and 7/8 variants were detected in 41 of 51 our diagnosed GS patients. Diagnostic value of the *UGT1A1* $(TA)_n$ marker was 80%.

In order to elucidate genetic basis of hyperbilirubinemia in remaining 20% of GS positive patients, identified with *UGT1A1* TA 6/6 and TA 6/7 repeats, we extended *UGT1A1* genetic testing. *UGT1A1* coding region with nearby intronic regions were sequenced. In only one patient with 6/7 TA repeats in promoter region of *UGT1A1* gene, two single nucleotide variants were found: NM_000463.2 c.997-82T>C (intron 2, position 602 of 683) and c.1084+12G>A (intron 3, position 12 of 283).

Population pharmacogenetic potential of *UGT1A1* $(TA)_n$ promoter genotypes in Serbia

Healthy control group had 67 pediatric individuals and 32 adults (median age 12.75, range 1.25 to 83 years). It consisted of 63 males (63%) and 37 females (37%).

The *UGT1A1* $(TA)_n$ promoter genotypes detected in the control group were TA 6/6, TA 6/7 and TA 7/7.

The distribution of *UGT1A1* $(TA)_n$ promoter genotypes in healthy control group in Serbia are given in Table 2. The frequencies of *UGT1A1* $(TA)_n$ promoter genotypes in healthy control group in Serbia were: TA 6/6 37%, TA 6/7 47% and TA 7/7 16%. Consequently, the frequency of the UGT1A1*28 allele is 40% in Serbia.

UGT1A1 $(TA)_n$ promoter genotypes detected in control group were in Hardy-Weinberg equilibrium (chi square test, $p=0.87$; exact test, $p=1$).

The differences in the distribution of *UGT1A1* $(TA)_n$ promoter genotypes between the control and GS patients' groups were statistically significant (Fisher test, $p<0.001$).

Discussion

Detection of number of TA repeats in *UGT1A1* promoter region using PCR amplification followed by acrylamide electrophoresis stained with Ag-nitrate and fragment length analysis methodology in pediatric GS patients and in healthy control group showed completely the same results. 10% of

the results were confirmed by DNA sequencing. Therefore, both methods used in our study can be considered as reliable and accurate. Acrylamide electrophoresis methodology is a time-consuming but its main advantage is being cost-effective. Using samples previously validated for number of TA repeats (TA 6/6 and TA 7/7) as controls, makes this approach sufficiently reliable for diagnostic purposes. Fragment analysis is fast and accurate but quite expensive. Sequencing analysis is relatively expensive and time-consuming but the most reliable of all methods used in this study. For that reason positive control samples were validated using DNA sequencing.

Numerous variants of *UGT1A1* gene, leading to elevated serum level of of non-conjugated bilirubin and development of several syndromes or diseases characterized with different levels of severity, have been reported. Some of these genetic variants, detected in homozygous or heterozygous state, give inactive *UGT1A1* alleles causing severe conditions, such as Crigler–Najjar type I or II syndromes [15]. The variants in *UGT1A1* gene also cause prolonged non-conjugated hyperbilirubinemia in the neonatal period, breast milk jaundice (BMJ) [15]. The mildest form of non-conjugated hyperbilirubinemia is GS. The most significant *UGT1A1* variant for development of GS is *UGT1A1*28,* with 7 TA repeats in the promoter region.

Our current study determined *UGT1A1* $(TA)_n$ promoter genotype distribution in pediatric GS patients in Serbia. As expected, 7/7 TA repeats in *UGT1A1* promoter (*UGT1A1*28* genotype) were found in majority of GS patients (76.47%), while the wild type 6/6 TA promoter repeats (*UGT1A1*1* genotype) was found in only two pediatric GS patients (3.92%).

GS non-risk *UGT1A1* (TA)n promoter genotypes (TA 6/6 and TA 6/7) were detected in 20% of our GS patients. Our results have shown that efficacy of the *UGT1A1* $(TA)_n$ promoter genotype variants as a diagnostic tool for GS is 80%, pointing out that genotyping of *UGT1A1**28 is not enough reliable genetic test for GS and that hypocaloric and phenobarbitone test cannot be replaced by this genetic test. In attempt to make UGT1A1 genetic test more reliable, we have decided to analyze coding and nearby intronic regions of *UGT1A1* gene in GS patients, carriers of non-risk GS alleles (TA 6/6 and TA 6/7). Surprisingly, only in one GS patient with TA 6/7 we have found additional variants, namely two intronic single nucleotide variants in heterozygous state, both of uncertain significance for GS development. Therefore, despite our finding that carriers of risk GS

genotypes have more than 21-fold higher odds for developing Gilbert syndrome than carriers of non-risk GS genotypes, genotyping of *UGT1A1* gene is not sufficient enough for diagnosis of GS.

For the Romanian cohort of GS individuals, the results showed that the 7/7 TA promoter genotype was identified in 32.33% of all subjects, the 6/7 TA promoter genotype was the most prevalent (57.64%) and the 6/6 TA promoter genotype was detected in 7.36% of the GS patients [25]. Another study reported that the prevalence of the *UGT1A1*28* allele in Valencia reached 87.6% among the patients referred for GS [26]. Our results demonstrated high concordance rate between clinical and genetic tests.

Our current study showed that the difference between levels of non-conjugated bilirubin at diagnosis were statistically significant between the non-risk GS carriers versus risk GS genotype carriers. In other words, the level of non-conjugated bilirubin was *UGT1A1* $(TA)_n$ promoter genotype-related in pediatric GS patients at diagnosis. After hypocaloric diet, the mean level of non-conjugated bilirubin was increased 2.91-fold in GS risk and 2.52-fold in GS non-risk genotype carriers. This increase was not *UGT1A1* $(TA)_n$ promoter genotype-related. Three-day hypocaloric diet test is still considered as one of important diagnostic tools for GS. The variation in bilirubin levels after a diet is considered positive for GS when the level of non-conjugated bilirubin increases by 100% over baseline. Nevertheless, a recent study revealed that the comparison between the results of the three-day hypocaloric diet test and the genetic study of gene *UGT1A1* show low association rate [26].

Our results also showed that, after a three-day phenobarbitone test, levels of non-conjugated serum bilirubin fracture decreased 27.4% in GS risk and 6.4% in GS non-risk genotype carriers in pediatric GS patients. The decrease in levels of non-conjugated bilirubin after phenobarbitone test comparing to levels of bilirubin before the test was *UGT1A1* $(TA)_n$ promoter genotype-related. It seems like the level of non-conjugated bilirubin drops more in GS risk genotype carriers comparing to the GS non-risk ones when applying phenobarbitone. Although the fact that GS risk genotypes contribute to hyperbilirubinemia more than GS non-risk genotypes, the treatment with phenobarbitone abolishes those differences leading to normal values of non-conjugated bilirubin.

Population pharmacogenomic research has shown that the study of pharmacogenomic markers in various populations is of great importance. Comprehensive data repositories that record the prevalence of clinically relevant genomic variants in populations worldwide, including pharmacogenomic biomarkers, are valuable tools that can be exploited not only to develop guidelines for medical prioritization, but most importantly, to facilitate integration of pharmacogenomics into health care systems and to support pre-emptive pharmacogenomic testing (27). *UGT1A1* $(TA)_n$ promoter genotypes resulting in decreased function of UGT1A1 enzyme are pharmacogenomic markers. Therefore, data on frequency of *UGT1A1* $(TA)_n$ promoter genotypes in particular populations are useful.

The frequency of the *UGT1A1*28* allele varies among ethnicities, being the highest in those of African (43%) and European (39%) origin and lowest in those of Asian (16%) origin [28,29]. Our results showed that the frequency of the *UGT1A1**28 allele in Serbia was 40%, which is in complete agreement with the data published for European populations.

Our study revealed that in Serbia the frequencies of *UGT1A1* TA 6/6, 6/7 and 7/7 promoter genotypes were as follows: 37%, 47% and 16%, respectively. This finding was similar with the literature data for Caucasian populations. In the population of the Republic of Macedonia the frequencies of *UGT1A1* 6/6, 6/7 and 7/7 TA promoter genotypes were 50%, 37.5% and 12.5% [30,31]. For healthy Croatian preschoolers the frequencies of *UGT1A1* 6/6, 6/7 and 7/7 TA promoter genotypes were 38.4%, 47.9% and 9.8% [32]. These data were similar for neighboring Slovenians, with the frequencies of *UGT1A1* $(TA)_n$ promoter genotypes as follows: 6/6 TA 38.1%, 6/7 TA 47.9%, 7/7 TA 13.6% [33,34]. Furthermore, in the Slovenian study it was confirmed that subjects with *UGT1A1* TA 7/7 promoter genotype had the highest and subjects with 6/6 TA promoter genotype the lowest total serum bilirubin levels. An Italian study analyzed *UGT1A1* $(TA)_n$ promoter genotypes among healthy subjects and the following results were reported: 43.9% were 6/6 TA promoter genotype carriers, 39.8% were 6/7 TA promoter genotype carriers and 16.3% were TA 7/7 promoter genotype carriers. In the same study the identified *UGT1A1* $(TA)_n$ promoter genotypes were correlated to serum bilirubin concentrations. The serum bilirubin concentrations were the highest in the Italian subjects with 7/7 TA promoter genotype, intermediate in the subjects who were 6/7 TA carriers and the lowest in the subjects with 6/6 TA

promoter genotype [35]. The unusual high frequency of *UGT1A1* TA 7/7 promoter genotype was reported for general Valencian population, reaching 32% [26].

Our finding that in Serbia the frequency of pharmacogenomic relevant *UGT1A1* promoter genotype (*UGT1A1**28/*UGT1A1**28) is 16%, points out that *UGT1A1* $(TA)_n$ promoter genotyping could be recommended for preemptive testing in Serbia. It is clinically confirmed that carriers of *UGT1A1* 7/7 TA promoter genotype treated with standard doses of irinotecan show an increased risk for developing hematological and/or digestive toxicities [36].

Keeping in mind that for some medications, such as atazanavir, the pharmacogenetic relevance of *UGT1A1* promoter genotypes should be considered even for heterozygous carriers (*UGT1A1**1/*UGT1A1**28), pharmacogenetic testing of this marker is even more important [37]. So, for 63% of Serbian patients to be treated with atazanavir, pre-emptive genetic testing for *UGT1A1**28 should be performed.

UGT1A1 genotyping is clinically beneficial. Genetic test can contribute to the confirmation of diagnosis in patients with elevated serum non-conjugated hyperbilirubinemia. However, genotyping of *UGT1A1* gene alone is not sufficient enough for diagnosis of GS, and cannot replace standard hypocaloric and phenobarbitone tests.

Furthermore, *UGT1A1* molecular genetic testing is important for individualization of therapy when drugs metabolized by UGT1A1 are administered. Enzyme function-decreased individuals are at risk for developing serious adverse drug reactions.

Finally, *UGT1A1* genotyping can enable the participation of patients with non-conjugated hyperbilirubinemia in clinical trials. High bilirubin levels disqualify these patients from many clinical trials. Atazanavir-associated hyperbilirubinemia has been described in GS patients administered with therapy for HIV [38] and in chronic myeloid leukemia treatment with nilotinib [39]. Hyperbilirubinemia has been reported in GS patients undergoing hepatitis C treatment [40]. However, if genetic testing shows that the patients are carriers of *UGT1A1* GS risk genotypes, they should not be excluded from clinical trials despite increased bilirubin values but should be treated taking into account *UGT1A1* $(TA)_n$ promoter genotype related higher bilirubin levels.

Acknowledgements

This work was supported by Ministry of Education, Science and Technological Development, Republic of Serbia (Grant No. III41004).

Declaration of Interest. The authors report no conflicts of interest.

References

1. Nagar S, Blanchard RL. Pharmacogenetics of Uridine diphosphoglucuronosyltransferase (UGT)1A family members and its role in patient response to irinotecan. Drug Metab Rev. 2006;38(3):393-409.

2. Barbarino JM, Haidar CE, Klein TE, Altman RB. PharmGKB summary: very important information for UGT1A1. Pharmacogenet Genomics. 2014;24(3):177-83.

3. Gong QH, Cho JW, Huang T, Potter C, Gholami N, Basu NK et al. Thirteen UDP-glucuronosyltransferase genes are encoded at the human UGT1 gene complex locus. Pharmacogenetics. 2001;11(4):357-68.

4. Strassburg CP, Kneip S, Topp J, Obermayer-Straub P, Barut A, Tukey RH et al. Polymorphic gene regulation and interindividual variation of UDP-glucuronosyltransferase activity in human small intestine. J Biol Chem. 2000;275(46):36164-71.

5. Strassburg CP, Nguyen N, Manns MP, Tukey RH. Polymorphic expression of the UDP-glucuronosyltransferase UGT1A gene locus in human gastric epithelium. Mol Pharmacol. 1998;54:647–654.

6. Wisnumurti D, Sribudiani Y, Porsch RM, Maskoen AM, Abdulhamied LI, Rahayuningsih SE et al. UGT1A1 Genetic Variations and Haplotype Associated with Neonatal Hyperbilirubinemia in Indonesian Population. Biomed Res Int. 2018;2018:9425843.

7. Marques SC, Ikediobi ON. The clinical application of UGT1A1 pharmacogenetic testing: Gene-enviroment interactions. Hum Genomics. 2010;4(4):238-49.

8. Bosma PJ, Chowdhury JR, Bakker C, Gantla S, de Boer A, Oostra BA, et al. The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. N Engl J Med. 1995; 333(18): 1171–5.

9. Aono S, Adachi Y, Uyama E, Yamada Y, Keino H, Nanno T, et al. Analysis of genes for bilirubin UDP-glucuronosyltransferase in Gilbert's syndrome. Lancet. 1995; 345(8955): 958-9.

10. Bosma P, Chowdhury JR, Jansen PH. Genetic inheritance of Gilbert's syndrome. Lancet. 1995; 346(8970): 314-5.

11. Radlović N. Hereditary hyperbilirubinemias. Srp Arh Celok Lek. 2014; 142(3-4): 257-60.

12. Radu P, Atsom J. Gilbert's syndrome-clinical and pharmacological implications. Isr Med Assoc J. 2001; 3: 593-8.

13. Radlović N, Leković Z, Mladenović M, Ristić D, Radlović V, Lekić V, et al. Gilbert's syndrome in children--our experience. Srp Arh Celok Lek. 2007; 135(5-6): 317-20.

14. Radlović N, Ristić D, Brdar R, Janić N, Leković Z, Janić D, et al. Association of hereditary elliptocytosis and Gilbert's syndrome as the cause of biliary calculosis: case report. Srp Arh Celok Lek. 2011; 139(5-6): 386-9.

15. Memon N, Weinberger BI, Hegyi T, Aleksunes LM. Inherited disorders of bilirubin clearance. Pediatr Res. 2016; 79(3): 378-86.

16. Pharmacogenomics Knowledge Base (https://www.pharmgkb.org) [accessed: 10.04.2018.]

17. Ha VH, Jupp J, Tsang RY. Oncology Drug Dosing in Gilbert Syndrome Associated with UGT1A1: A Summary of the Literature. Pharmacotherapy. 2017; 37(8): 956-972.

18. de Morais SM, Uetrecht JP, Wells PG. Decreased glucuronidation and increased bioactivation of acetaminophen in Gilbert's syndrome. Gastroenterology. 1992; 102(2): 577-86.

19. Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group. Recommendations from the EGAPP Working Group: can UGT1A1 genotyping reduce morbidity and mortality in patients with metastatic colorectal cancer treated with irinotecan? Genet Med. 2009; 11(1): 15–20.

20. Zucker SD, Qin X, Rouster SD, Yu F, Green RM, Keshavan P, et al. Mechanism of indinavir-induced hyperbilirubinemia. Proc Natl Acad Sci U S A. 2001; 98(22): 12671-6.

21. Radojkovic D, Kusic J. Silver staining of denaturing gradient gel electrophoresis gels. Clin Chem. 2000; 46(6 Pt 1): 883-4.

22. Ribrag V, Koscielny S, Casasnovas O, Cazeneuve C, Brice P, Morschhauser F et al. Pharmacogenetic study in Hodgkin lymphomas reveals the impact of UGT1A1 polymorphisms on patient prognosis. Blood. 2009; 113(14): 3307-13.

23. Karan-Djurasevic T, Palibrk V, Kostic T, Spasovski V, Nikcevic G, Srzentic S et al. Mutational status and gene repertoire of IGHV-IGHD-IGHJ rearrangements in Serbian patients with chronic lymphocytic leukemia. Clin Lymphoma Myeloma Leuk. 2012; 12(4): 252-60.

24. Wigginton JE, Cutler DJ, and Abecasis GR. A note on exact tests of Hardy-Weinberg equilibrium. Am J Hum Genet. 2005; 76(5): 887-93.

25. Radoi VE, Ursu RI, Poenaru E, Arsene C, Bohiltea CL, Bohiltea R. Frequency of the UGT1A1*28 Polymorphism in a Romanian Cohort of Gilbert Syndrome Individuals. J Gastrointestin Liver Dis. 2017; 26(1): 25-28.

26. Torres AK, Escartín N, Monzó C, Guzmán C, Ferrer I, González-Munoz C, et al. Genetic susceptibility to Gilbert's syndrome in a Valencian population; efficacy of the fasting test. Rev Clin Esp. 2017; 217(1): 1-6.

27. Viennas E, Komianou A, Mizzi C, Stojiljkovic M, Mitropoulou C, Muilu J et al. Expanded national database collection and data coverage in the FINDbase worldwide database for clinically relevant genomic variation allele frequencies. Nucleic Acids Res. 2017 Jan 4;45(D1):D846-D853.

28. Beutler E, Gelbart T, Demina A. Racial variability in the UDP glucuronosyltransferase 1 (UGT1A1) promoter: a balanced polymorphism for regulation of bilirubin metabolism? Proc Natl Acad Sci U S A 1998; 95(14): 8170–4.

29. Premawardhena A, Fisher CA, Liu YT, Verma IC, de Silva S, Arambepola M, et al. The global distribution of length polymorphisms of the promoters of the glucuronosyltransferase 1 gene (UGT1A1): hematologic and evolutionary implications. Blood Cells Mol Dis. 2003; 31(1): 98–101.

30. Bajro MH, Josifovski T, Panovski M, Jankulovski N, Nestorovska AK, Matevska N et al. Promoter length polymorphism in UGT1A1 and the risk of sporadic colorectal cancer. Cancer Genet. 2012;205(4):163-7, 11.

31. Kapedanovska Nestorovska A, Jakovski K, Naumovska Z, Hiljadnikova Bajro M, Sterjev Z, Eftimov A, et al. Distribution of the most Common Genetic Variants Associated with a Variable Drug Response in the Population of the Republic of Macedonia. Balkan J Med Genet. 2015;17(2):5-14.

32. Marinković N, Pasalić D, Grsković B, Ferencak G, Honović L, Rukavina AS. Genotype frequencies of UDP-glucuronosyltransferase 1A1 promoter gene polymorphism in the population of healthy Croatian pre-scholars. Coll Antropol. 2008; 32(3): 725-9.

33. Mlakar SJ, Ostanek B. Development of a new DHPLC assay for genotyping UGT1A (TA)n polymorphism associated with Gilbert's syndrome. Biochem Med (Zagreb). 2011; 21(2): 167-73.

34. Ostanek B, Furlan D, Mavec T, Lukac-Bajalo J. UGT1A1(TA)n promoter polymorphism—a new case of a (TA)8 allele in Caucasians. Blood Cells Mol Dis. 2007; 38(2): 78-82.

35. Biondi ML, Turri O, Dilillo D, Stival G, Guagnellini E. Contribution of the TATA-box genotype (Gilbert syndrome) to serum bilirubin concentrations in the Italian population. Clin Chem. 1999; 45(6 Pt 1): 897-8.

36. Etienne-Grimaldi MC, Boyer JC, Thomas F, Quaranta S, Picard N, Loriot MA, et al. UGT1A1 genotype and irinotecan therapy: general review and implementation in routine practice. Fundam Clin Pharmacol. 2015; 29(3): 219-37.

37. Gammal RS, Court MH, Haidar CE, Iwuchukwu OF, Gaur AH, Alvarellos M et al; Clinical Pharmacogenetics Implementation Consortium. Clinical Pharmacogenetics Implementation Consortium (CPIC) Guideline for UGT1A1 and Atazanavir Prescribing. Clin Pharmacol Ther. 2016;99(4):363-9.

38. Culley CL, Kiang TK, Gilchrist SE, Ensom MH. Effect of the UGT1A1∗28 allele on unconjugated hyperbilirubinemia in HIV-positive patients receiving Atazanavir: a systematic review. Ann Pharmacother. 2013; 47(4): 561–72.

39. Fujita K, Sugiyama M, Akiyama Y, Ando Y, Sasaki Y. The small-molecule tyrosine kinase inhibitor nilotinib is a potent noncompetitive inhibitor of the SN-38 glucuronidation by human UGT1A1. Cancer Chemother Pharmacol. 2011; 67(1): 237–41.

40. de Souza MM, Vaisberg VV, Abreu RM, Ferreira AS, da Silva Ferreira C, Nasser PD, et al. UGT1A1∗28 relationship with abnormal total bilirubin levels in chronic hepatitis C patients: Outcomes from a case–control study. Medicine (Baltimore). 2017; 96(11): e6306.