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ORIGINAL ARTICLE

UGT1A1 $(TA)_n$ PROMOTER GENOTYPE: DIAGNOSTIC AND POPULATION PHARMACOGENETIC MARKER IN SERBIA

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ABSTRACT

The UGT1A1 enzyme is involved in the metabolism of bilirubin and numerous medications. Unconjugated hyperbilirubinemia, commonly presented as Gilbert syndrome (GS), is a result of decreased activity of the UGT1A1 enzyme, variable number of TA repeats in the promoter of the *UGT1A1* gene affects enzyme activity. Seven and eight TA repeats cause a decrease of UGT1A1 activity and risk GS alleles, while six TA repeats contribute to normal UGT1A1 activity and non-risk GS allele. Also, the *UGT1A1* $(TA)_n$ promoter genotype is recognized as a clinically relevant pharmacogenetic marker. The aim of this study was to assess diagnostic value of *UGT1A1* $(TA)_n$ promoter genotyping in pediatric GS patients. Correlation of the *UGT1A1* $(TA)_n$ genotypes and level of unconjugated 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 methodologies, polymerase chain reaction (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 80.0% of patients. Therefore, *UGT1A1* $(TA)_n$ promoter genotyping is not a 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 was 63.0%, pointing out that *UGT1A1* $(TA)_n$ promoter genotyping could be recommended for preemptive pharmacogenetic testing in Serbia.

Keywords: Gilbert syndrome (GS); Unconjugated hyperbilirubinemia; Population pharmacogenetics, *UGT1A1* $(TA)_n$ promoter variants.

INTRODUCTION

Uridin diphospho-glucuronosyl transferases (UGT) are a 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]. The human UGT superfamily is divided into four families: UGT1A, UGT2, UGT3 and UGT8 [2]. The UGT1A family is encoded by the *UGT1A* gene complex located on chromosome 2q37. This gene complex at the 5' region has 13 variable exons, linked to four common exons at the 3' region of the *UGT1A* gene. Each of the 13 5' exons has its own TATA promoter elements. Four of 13 first exons are pseudoexons, and nine exons are viable, independently transcribed as the first exon. Those nine different first exons with highly conserved regions from exon 2 to exon 5, generate nine different transcripts (UGT1A1, UGT1A3 through UGT1A10). Each transcript has identical 3' ends and a unique 5' end. Variable first exon provides substrate specificity, while highly conserved common exons contain

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sites for interactions with UDP glucuronic acid as common substrate [3]. The *UGT1A1* gene is expressed in the liver, colon, intestine and stomach [4,5]. The UGT1A1 enzyme has a main role in glucuronidation of bilirubin. There are 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 (GS), 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 the *UGT1A1* gene are different numbers 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 the *UGT1A1* gene and, consequently, the activity of the UGT1A1 enzyme, depends on the number of TA repeats. A higher number of TA repeats causes decreased enzyme activity. Heterozygous status of *UGT1A1**28 results in 25.0% decreased enzyme activity and homozygous status of this variant reduced transcription activity by 70.0% [7]. This leads to GS, a mild form of intermittent unconjugated hyperbilirubinemia, that lacks hemolysis or hepatocellular injury. Therefore, six TA repeats in the *UGT1A1* gene promoter is considered as non-risk GS allele and seven or eight TA repeats are responsible for development of GS (risk GS allele).

Gilbert syndrome is a benign hereditary condition, commonly presented in 3.0-13.0% in the general population. Gilbert syndrome is inherited in an autosomal-recessive manner, but several cases have been reported as dominantly inherited [10,11]. Gilbert syndrome 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].

The *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 unconjugated bilirubin further contributes to development of GS.

Gilbert syndrome 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 cosmetic 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 the *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 a premature stop codon have been described. Lack of UGT1A1 activity leads to a high level of bilirubin shortly after birth (20.0-50.0 mg/dL), causing bilirubin encephalopathy, kernicterus and death. Crigler-Najjar syndrome type 1 could be managed by repeated exchange transfusions, long-term phototherapy, plasmapheresis, hemoperfusion and finally, the only successful therapy is liver transplantation. Several single nucleotide variations in the *UGT1A1* gene have been reported to cause substitution of a single amino acid and reduce enzyme activity to less than 10.0% of normal activity, but total bilirubin levels do not exceed 20.0 mg/dL. This condition is recognized as a Crigler-Najjar syndrome type 2 [15]. In this case, optimal therapy is phenobarbital that stimulates UGT1A1 activity.

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

The presence of seven or eight TA repeats in the promoter of the *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 have already been applied in routine clinical practice [19].

The aim of this study was to analyze if *UGT1A1* $(TA)_n$ promoter variants can be efficient diagnostic tools for easier and faster diagnosis of GS, by using accurate and cost-effective methodology for detection of number of TA repeats in the *UGT1A1* promoter. Correlation of *UGT1A1* $(TA)_n$ promoter genotypes, risk GS and non-risk GS, with levels of unconjugated 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 that the 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 the University Children's Hospital, Belgrade, Serbia. Ethical approval was obtained from the Ethics Committee of University Children's Hospital, University of Belgrade. The 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 5 mL 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. The 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 a blood sample was taken during routine pediatric check-ups. A 5 mL 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 the GS patients group at diagnosis and after a 3-day hypocaloric diet test (400 kcal per day) during standard laboratory examinations. Further, a 3-day phenobarbitone test (2 mg/kg/day) was performed in patients group and levels of conjugated and total bilirubin was measured also. Level of unconjugated bilirubin was calculated as a mathematical difference between the total and conjugated levels of bilirubin.

***UGT1A1* $(TA)_n$ Promoter Genotyping.** DNA was extracted from blood samples using the QIAamp® DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). The number of TA repeats in the *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, Burlington, ON, Canada), 1 × PCR reaction buffer (Qiagen GmbH), 1 × Q solution (Qiagen GmbH), 2.75 mM $MgCl_2$, 1 U HotStar DNA polymerase (Qiagen GmbH). 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 seconds denaturation at 95 °C, 30 seconds annealing at 63 °C, and 30 seconds elongation at 72 °C, ending with a final extension period of 7 min. at 72 °C. The PCR fragments were visualized on 2.0% agarose gel and finally analyzed using 15.0% acrylamide electrophoresis (19:1 acrylamide/bisacrylamide in 1 × TBE buffer, run at 300V and 60 mA for 5 hours) 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 the 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 50 ng of DNA, 1 × PCR buffer (Qiagen GmbH), 15.4 mM $MgCl_2$, 0.4 mM dNTPs, 1 U of HotStar Polymerase (Qiagen GmbH) and 0.4 μM of both UGT1A1F (FAM dye-labeled, 5'-famTAC AGT CAC GTG ACA CAG-3') and UGT1A1R (5'-TTT GCT CCT GCC AGA GGT TCG-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 seconds denaturation at 95 °C, 30 seconds annealing at 55 °C, and 30 seconds elongation at 72 °C, ending with a final extension period of 7 min. at 72 °C. The PCR products were separated on an ABI PRISM® 3130 DNA analyzer (Applied Biosystems, Foster City, CA, USA) and the collected data were analyzed with the GeneMapper version 4 software (Applied Biosystems).

Ten percent of samples were randomly chosen and results of *UGT1A1* $(TA)_n$ promoter genotyping by the PCR/acrylamide electrophoresis methodology were checked and

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

Primer	Sequences (5'>3')	Annealing Temperature (°C)
UGT1A1: ex1-1F	GCT ACC TTT GTG GAC TGA CAG C	60
UGT1A1: ex1-1R	CCA TGA GCT CCT TGT TGT GCA G	
UGT1A1: ex1-2F	GCC ATT CCA AAG GGA GGA TG	57
UGT1A1: ex1-2R	GAT GAT GCC AAA GAC AGA CTC AAA C	
UGT1A1: int1ex2F	CTG TAA GCA GGA AAC CCT TCC TC	58
UGT1A1: int1ex2R	GGA TTA ATA GTT GGG AAG TGG CAG G	
UGT1A1: ex3F	AAG TTG CCA GTC CTC AGA AGC	60
UGT1A1: ex3R	TGT TAC TCA CAT GCC CTT GCA G	
UGT1A1: ex4int4F	TGC AAG GGC ATG TGA GTA ACA C	58
UGT1A1:ex4int4R	GCA CTC CAG CCT AGG TGA C	
UGT1A1: ex5F	CAG GTT TCC TTT CCC AAG TTT GG	58
UGT1A1: ex5R	CAC TCT GGG GCT GAT TAA TTT ATG C	

confirmed using Sanger sequencing methodology. Those samples were used as positive/negative controls on each acrylamide electrophoresis run [23].

***UGT1A1* Gene Analysis.** According to ENST00000 305208.9, primers were designed to analyze all five coding exons and nearby intronic sequences of the *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), 1 × PCR reaction buffer, 1.4 mM $MgCl_2$, 1 U DNA polymerase (KAPA Biosystems, Wilmington, MA, 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 seconds denaturation at 95 °C, 30 seconds annealing as given in Table 1, and 30 seconds elongation at 72 °C, ending with a final extension period of 10 min. at 72 °C. The PCR fragments were visualized on 2.0% agarose gel, separated on an ABI PRISM® 3130 DNA analyzer (Applied Biosystems) and the collected data were analyzed with the Gene Mapper version 4 software (Applied Biosystems).

Statistical Analysis. All statistical analysis was performed using the SPSS® Statistics version 21 software (IBM). 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 the control group were checked for the Hardy-Weinberg equilibrium by exact test [24]. The distribution of unconjugated bilirubin in GS patients was checked for normality using Shapiro-Wilk and Kolmogorov-Smirnov tests. The difference in the level of unconjugated bilirubin between carriers of risk and non-risk GS genotypes was assessed using the Mann-Whitney test. Probability values of <0.05 were considered statistically significant. All tests were nondirectional (two-tailed).

RESULTS

***UGT1A1* $(TA)_n$ Promoter Genotyping as a Diagnostic Tool for Gilbert Syndrome.** The GS patient 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.

The detection of the number of TA repeats in *UGT1A1* promoter using PCR amplification followed by 15.0% 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,

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

GS Patients (n = 51)			Controls (n = 100)		
Repeats	n	%	Repeats	n	%
6/6	2	3.92	6/6	37	37.0
6/7	8	15.87	6/7	47	47.0
7/7	39	76.47	7/7	16	16.0
7/8	2	3.92	7/8	0	0.0

Table 3. Levels of unconjugated bilirubin in pediatric Gilbert syndrome patients.

Parameters	Unconjugated Bilirubin in GS Risk Group (μmol/L)[a]	Unconjugated Bilirubin in GS Non-Risk Group (μmol/L)[a]	*p* Values[b]
At diagnosis	27.51 ± 19.16	16.92 ± 7.57	0.079
After hypocaloric test	62.02 ± 43.85	41.40 ± 14.67	0.240
After phenobarbitone test	18.01 ± 14.57	14.23 ± 6.71	0.626

GS: Gilbert syndrome.
[a] The results are presented as mean ± standard deviations.
[b] The *p* values were assessed using the Mann-Whitney test.

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%).

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* 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 had more than 21-fold higher odds for developing GS 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, $p < 0.05$).

The difference between levels of unconjugated bilirubin at diagnosis in pediatric GS patients were statistically significant between the non-risk GS genotype carriers and

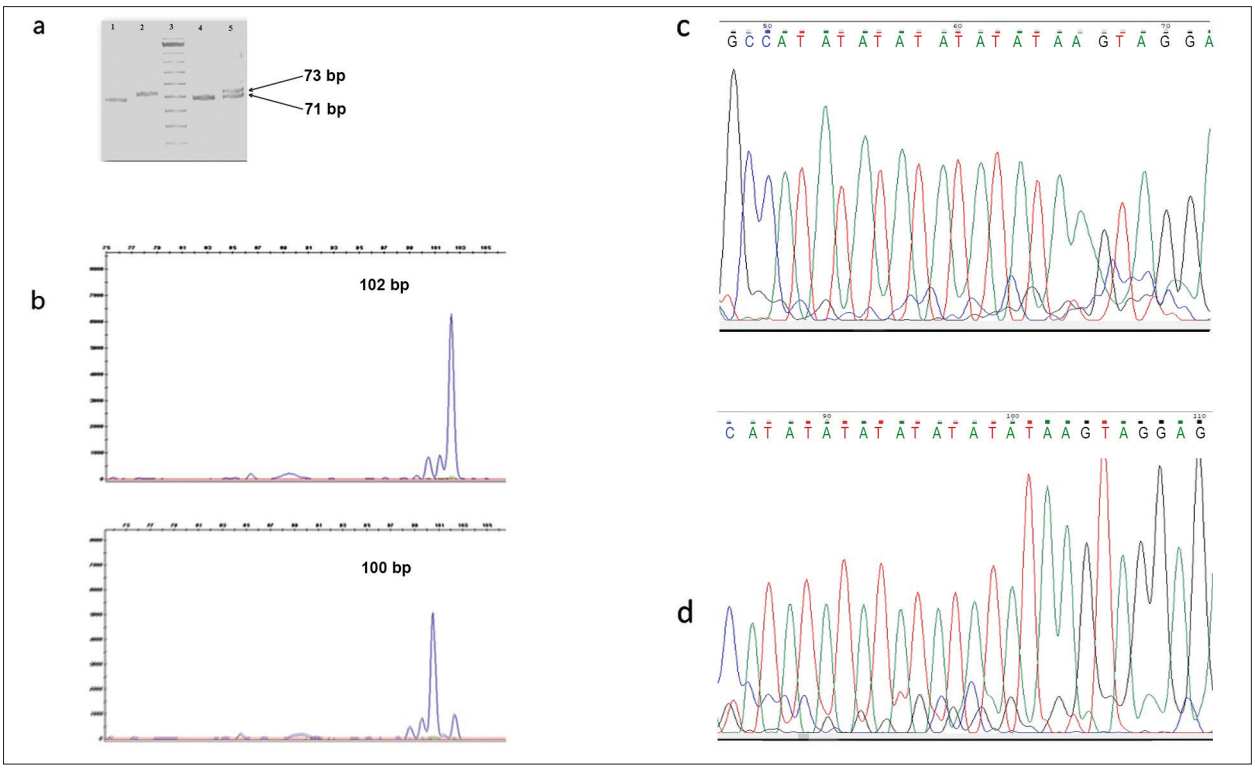

Figure 1. (a) *UGT1A1* $(TA)_n$ promoter PCR products on 15.0% acrylamide gel electrophoresis stained with Ag-nitrate. Wells 1 and 4: 6/6 TA repeats (71 bp); well 2: 7/7 TA repeats (73 bp); well 5: 6/7 TA repeats (71 and 73 bp); well 3: 5 bp DNA ladder. (b) Electophoretograms obtained with fragment length analysis of *UGT1A1* $(TA)_n$ promoter repeats. The upper peak is 7/7 TA repeats (102 bp); the lower peak is 6/6 TA repeats (100 bp). (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.

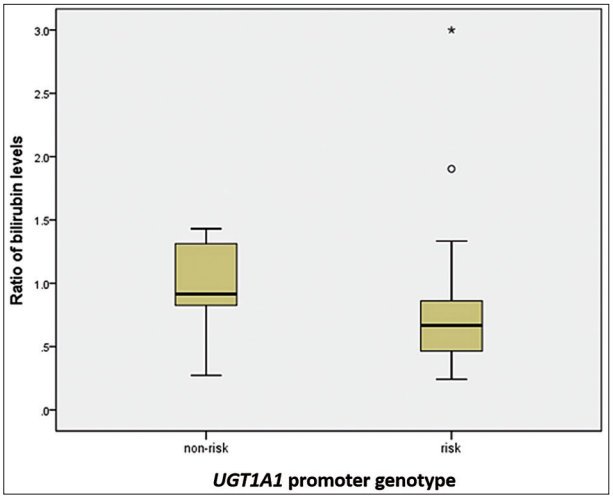

Figure 2. Median and interquartile range of ratios between levels of unconjugated bilirubin after phenobarbitone test comparing to levels of bilirubin before the test of unconjugated bilirubin according to GS risk groups (GS non-risk, GS risk) (Mann-Whitney test, $p = 0.040$).

risk GS genotype carriers (Mann-Whitney test, $p = 0.079$). After hypocaloric diet, the mean level of unconjugated 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 3-day phenobarbitone test, levels of total and conjugated bilirubin were also measured in pediatric GS patients. The unconjugated serum bilirubin fracture decreased 27.4% in GS risk and 6.4% in GS non-risk genotype carriers. The difference of a decrease of unconjugated bilirubin levels after the 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).

UGT1A1 TA 7/7 and 7/8 variants were detected in 41 of 51 of our diagnosed GS patients. Diagnostic value of the *UGT1A1* $(TA)_n$ marker was 80.0%. In order to elucidate the genetic basis of hyperbilirubinemia in the remaining 20.0% GS-positive patients, identified with *UGT1A1* TA 6/6 and TA 6/7 repeats, we extended the *UGT1A1* genetic testing. The *UGT1A1* coding region with nearby intronic regions were sequenced. In only one patient with 6/7 TA repeats in promoter region of the *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. The 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 (63.0%) and 37 females (37.0%). 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 the healthy control group in Serbia were: TA 6/6 37.0%, TA 6/7 47.0% and TA 7/7 16.0%. Consequently, the frequency of the UGT1A1*28 allele is 40.0% in Serbia.

The *UGT1A1* $(TA)_n$ promoter genotypes detected in the control group were in Hardy-Weinberg equilibrium (χ^2 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 the number of TA repeats in the *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 a healthy control group showed completely the same results. Ten percent 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 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 the *UGT1A1* gene, leading to elevated serum level of of unconjugated 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 states, give inactive *UGT1A1* alleles causing severe conditions, such as Crigler-Najjar type 1 or 2 syndromes [15]. The variants in *UGT1A1* gene also cause prolonged unconjugated hyperbilirubinemia in the neonatal period, breast milk jaundice (BMJ) [15]. The mildest form of unconjugated hyperbilirubinemia is GS. The most significant *UGT1A1* variant for development of GS is *UGT1A1 *28,* with seven 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 the 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%).

Gilbert syndrome non-risk *UGT1A1* $(TA)_n$ promoter genotypes (TA 6/6 and TA 6/7) were detected in 20.0% 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 80.0%, pointing out that genotyping of *UGT1A1**28 is not enough of a reliable genetic test for GS, and that hypocaloric diet and phenobarbitone tests cannot be replaced by this genetic test. In an attempt to make the UGT1A1 genetic test more reliable, we decided to analyze coding and nearby intronic regions of the *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 did we find additional variants, namely two intronic single nucleotide variants in a heterozygous state, both of uncertain significance for GS development. Therefore, despite our finding that carriers of the risk GS genotypes have more than 21-fold higher odds for developing GS than carriers of non-risk GS genotypes, genotyping of the *UGT1A1* gene is not sufficiently reliable 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% in the patients referred for GS [26]. Our results demonstrated a high concordance rate between clinical and genetic tests.

Our current study showed that the difference between levels of unconjugated bilirubin at diagnosis were statistically significant between the non-risk GS carriers *vs*. risk GS genotype carriers. In other words, the level of unconjugated bilirubin was *UGT1A1* $(TA)_n$ promoter genotype-related in pediatric GS patients at diagnosis. After hypocaloric diet, the mean level of unconjugated 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. The 3-day hypocaloric diet test is still considered as one of the important diagnostic tools for GS. The variation in bilirubin levels after a diet is considered positive for GS when the level of unconjugated bilirubin increases by 100.0% over baseline. Nevertheless, a recent study revealed that the comparison between the results of the 3-day hypocaloric diet test and the genetic study of the *UGT1A1* gene show a low association rate [26].

Our results also showed that, after a 3-day phenobarbitone test, levels of unconjugated 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 unconjugated 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 unconjugated 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 unconjugated 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 preemptive pharmacogenomic testing [27]. *UGT1A1* $(TA)_n$ promoter genotypes resulting in decreased function of the 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 (43.0%) and European (39.0%) origin and lowest in those of Asian (16.0%) origin [28,29]. Our results showed that the frequency of the *UGT1A1**28 allele in Serbia was 40.0%, 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: 37.0, 47.0 and 16.0%, 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 50.0, 37.5.0 and 12.5% [30,31]. For healthy Croatian preschoolers, the frequencies of the *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 the *UGT1A1* TA 7/7 promoter genotype had the highest and subjects with the 6/6 TA promoter genotype the lowest total serum bilirubin levels. An Italian study analyzed *UGT1A1* $(TA)_n$ promoter genotypes in 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 unusually high frequency of *UGT1A1* TA 7/7 promoter genotype was reported for general Valencian population, reaching 32.0% [26].

Our finding that in Serbia the frequency of pharmacogenomic relevant *UGT1A1* promoter genotype (*UGT1A1* *28/*UGT1A1**28) is 16.0%, points out that the *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 genotypes 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]. Thus, for 63.0% of Serbian patients to be treated with atazanavir, preemptive 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 unconjugated hyperbilirubinemia. However, genotyping of the *UGT1A1* gene alone is not sufficient 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 unconjugated hyperbilirubinemia in clinical trials. High bilirubin levels disqualify these patients from many clinical trials. Atazanavir-associated hyperbilirubinemia has been described in GS patients receiving 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.

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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