Rare TA repeats in promoter TATA box of the UDP glucuronosyltranferase (UGT1A1) gene in Croatian subjects

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Abstract

Background: Gilbert’s syndrome is a chronic or recurrent mild unconjugated hyperbilirubinemia caused by decreased activity of UDP glucuronosyltranferase (UGT1A1). The most common cause of Gilbert’s syndrome in Caucasians is homozygous variant of the A(TA)nTAA promoter polymorphism. Alleles with five or eight TA repeats have also been described, but they are very rare in Caucasian populations.

Methods: Over a 6-year period (2001–2006), 1109 subjects with suspected Gilbert’s syndrome were included in this study. Genotyping of (TA)n and (TA)n alleles was performed using high-resolution electrophoretic separation of amplified PCR products on Spreadex EL300 gels. In seven subjects, aberrant electrophoretic patterns were observed and additionally sequenced on an ABI Prism 310 Genetic Analyzer.

Results: Genotype distributions for 1102 subjects with (TA)n or (TA)n alleles were as follows: 54.10%, 26.33% and 18.49% for the (TA)n/(TA)n, (TA)n/(TA)n and (TA)n/(TA)n respectively. Sequencing of seven samples that could not be identified as one of these alleles identified four subjects with the (TA)n/(TA)n, two with the (TA)n/(TA)n and one with the (TA)n/(TA)n genotype.

Conclusion: Genotyping of TA repeats in the promoter region of the UGT1A1 gene revealed the presence of rare alleles with five or eight TA repeats, with a very high frequency of the (TA)n allele in subjects suspected of having Gilbert’s syndrome.


Keywords: genetic polymorphism; Gilbert’s syndrome; hyperbilirubinemia; neonatal jaundice.

Introduction

Gilbert’s syndrome (GS) is an inherited, chronic, intermittent, mild, unconjugated hyperbilirubinemia without liver disease and overt hemolysis, caused by decreased activity of UDP glucuronosyltranferase (UGT1A1) (1–3). Bilirubin concentration typically fluctuates with time, ranging from 20 to 50 μmol/L [rarely over 85 μmol/L (3)], with an increase after stress, infection or starving. Other liver functions are normal, and GS does not require any special medical treatment. However, the utmost importance of recognizing this condition lies in differential diagnostics from other liver dysfunctions associated with hyperbilirubinemia. UGT1A1 genotyping can also be useful in neonatal diagnostics, as it was found to be associated with prolonged neonatal hyperbilirubinemia (4). Although very rarely, GS may coincide with some hereditary anemia, such as hereditary spherocytosis (5), congenital dyserythropoietic anemia (6) and glucose-6-phosphate dehydrogenase deficiency (7), and increase the risk of gallstone formation.

The decreased activity of UGT1A1 may be caused by numerous polymorphisms in the UGT1A1 gene (8). The most common polymorphism in Caucasian populations is a TA insertion in TA(TA)nTAA sequence in the promoter region (9). This allele, (TA)5, is responsible for a decreased transcription rate and results in 30% lower activity compared to the wild type. Because bilirubin conjugation catalyzed by UGT1A1 appears to be the rate-limiting step in bilirubin elimination, decreased enzymatic activity in GS leads to lower bilirubin conjugation and its elevated blood concentrations.

Promoter TATA box is highly polymorphic and variants with five or eight TA repeats were also described in association with GS. The number of the repeats inversely correlates with transcriptional activity (10), thus allele (TA)n is found to be associated with the lowest and allele (TA)n with the highest enzyme activity.

The distribution of TATA box polymorphisms shows a large ethnic variability (9). In comparison to other ethnic groups, the frequency of the (TA)n allele in Caucasian populations is intermediate (38.7%). Asian populations show the lowest frequency of the (TA)n allele (16%), yet it is still the most common cause of GS. The highest frequency was found in Africans (49.5%), although their serum bilirubin concentration was in general 15%–20% lower than in Caucasians (11). Interestingly, in Africans the alleles (TA)n and (TA)n were found to be 3.5% and 6.9%, respectively (9, 12), while these alleles are very rare in Caucasian populations. However, the literature
describing possible associations of these alleles with GS in Caucasian populations is scarce.

Hereby we report the frequencies of UGT1A1 promoter TA/(TA)nTAA polymorphisms in Croatian subjects with clinically suspected GS, the relation to bilirubin level and phenotype of seven subjects with rare (TA)6 and (TA)8 alleles. Up to now, (TA)6 and (TA)8 alleles have not been described in the Croatian population.

Materials and methods

Subjects

This retrospective study included all subjects genotyped for the UGT1A1 polymorphism in the University Department of Chemistry, Sestre Milosrdnice University Hospital in Zagreb, over a 6-year period (2001–2006). The total cohort consisted of 1109 Croatian subjects (571 males; age median: 14 years, interquartile range: 8–21 years; and 538 females; age median: 14 years, interquartile range: 11–18 years). UGT1A1 genotyping was performed due to several reasons: prolonged hyperbilirubinemia, hyperbilirubinemia after stress or starving, excessive neonatal hyperbilirubinemia, breast milk jaundice or chronic hepatic diseases. The subjects were hospital patients and outpatients from the entire geographic region of Croatia, as during the 6-year study period Sestre Milosrdnice University Hospital was the only center in Croatia that performed UGT1A1 genotyping. The cohort also included family members of the examined subjects as a part of a family study.

This study was approved by the Ethic Committee of the hospital.

Samples

Samples were collected after overnight fasting – whole blood with K3EDTA for genotyping and serum for bilirubin level determination and all analyses were performed immediately after the collection. Total bilirubin concentration was determined on a Olympus AU2700 Analyzer using Olympus reagents (Olympus, Hamburg, Germany). The rifampicin test was performed in adolescents and adults by measuring bilirubin levels before and 4 h after single peroral administration of 600 mg rifampicin. The test was defined as positive if bilirubin level was elevated after rifampicin dose.

Hyperbilirubinemia was defined as bilirubin level > 20.5 μmol/L.

Genotyping

DNA isolation was performed using the salting out method according to Lahiri and Nurnberger (13) or by a commercially available kit QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). UGT1A1 polymorphisms were determined according to the modified method developed by Sampietro et al. (14). In brief, PCR fragment was amplified using forward (5'-TAACTTGTTGATCGATTGTTTTTG-3') and reverse (5'-ACAGCCATGCGCTTGTGCT-3') primers. In PCR, negative and positive control samples ([TA]6 and [TA]7) were also amplified. PCR fragments of 90 bp, (TA)6, or 92 bp, (TA)8, were separated by high-resolution electrophoresis on commercial Spreadex EL300 gels (Elchrom Scientific, Cham, Switzerland) for 4 h at 100 V. The external quality assessment was performed by DGKL (United Germany Society of Clinical Chemistry and Laboratory Medicine).

DNA sequencing analysis

The sequencing analyses were performed at the Department of Biochemistry and Molecular Biology, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb.

Samples that showed electrophoretic patterns different from the (TA)6 and (TA)8 alleles (n = 7) and six randomly chosen samples containing the (TA)6 and (TA)8 alleles were additionally sequenced. PCR products (253 bp for the wild type) were amplified using forward (5'-AAGTGAACCTCCC-TGCTACCTT-3') and reverse (5'-CCACTGGATACACAGTA-TCT-3') primers (15). After purification of the fragments with a QIAquick PCR Purification Kit (Qiagen), the nucleotide sequences were analyzed with a DNA Sequencing Big Dye Terminator v3.0 Kit on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions. The data were analyzed using Sequencing Analysis software®, Version 3.4 (Applied Biosystems, USA). For effective removal of excess DyeDeoxy™ terminators (Big Dye™ Terminator, Version 3.1 Ready Reaction Cycle Sequencing Kit; Applied Biosystems Carrington, UK) from completed DNA sequencing reaction prior to the analysis on the ABI Prism 310 Genetic Analyzer, Centri-Sep™ Spin Columns (Princeton Separation, Adelphia, NJ, USA) were used.

Statistical analysis

Genotype frequencies were calculated by direct counting. Differences between observed and expected genotype frequencies for calculating Hardy-Weinberg equilibrium were tested using the χ2-test. Normality of distributions for quantitative variables (age and bilirubin level) was tested using the Kolmogorov-Smirnov test. Variables were not distributed normally and are presented as median and interquartile range. Differences in bilirubin concentrations across different genotype groups were tested with analysis of variance on ranks. Dunn’s method was used for post hoc testing between specific genotypes. A p-value < 0.05 was considered statistically significant. Data were analyzed using the statistical software SigmaStat for Windows Version 3.00 (SPSS Inc, Chicago, IL, USA).

Results

Detected genotype and allelic frequencies of UGT1A1 promoter polymorphisms are shown in Tables 1 and 2. Genotype frequencies were found to be in Hardy-Weinberg equilibrium (p = 0.321). (TA)6/(TA)7 (54.10%) was found to be the most frequent genotype in the studied cohort. For the first time, rare polymorphisms in the UGT1A1 promoter region associated with GS were detected in Croats: (TA)6 in four subjects present as (TA)6/(TA)6, and (TA)8 in three subjects.

| Table 1 Detected genotype frequencies of UGT1A1 polymorphisms. |
|---------------|----------------|---------|
| Genotype      | n = 1109       | %       |
| (TA)6/(TA)6   | 210            | 18.94   |
| (TA)6/(TA)7   | 292            | 26.33   |
| (TA)7/(TA)7   | 600            | 54.10   |
| (TA)6/(TA)8   | 4              | 0.36    |
| (TA)7/(TA)8   | 1              | 0.09    |
| (TA)8/(TA)8   | 2              | 0.18    |
Table 2 Detected allelic frequencies of UGT1A1 polymorphisms.

<table>
<thead>
<tr>
<th>Allele</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>(TA)$_6$</td>
<td>4</td>
<td>0.18</td>
</tr>
<tr>
<td>(TA)$_5$</td>
<td>713</td>
<td>32.15</td>
</tr>
<tr>
<td>(TA)$_7$</td>
<td>1498</td>
<td>67.54</td>
</tr>
<tr>
<td>(TA)$_8$</td>
<td>3</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Discussion

In the studied cohort, the most common allele was (TA)$_7$ (67.54%). Clinical suspicion of GS was confirmed in 54.10% [600 subjects with (TA)$_7$/(TA)$_7$]. The other part of the cohort included family members of affected subjects without GS, and subjects in which the final diagnosis has yet to be defined. Because only polymorphisms in TATA box of the UGT1A1 gene were genotyped, we could not exclude one of the numerous other polymorphisms to be the cause of GS in these subjects.

Bilirubin levels were moderately elevated in patients with GS. Mean value of total bilirubin was 29.4 $\mu$mol/L, which is in accordance with other studies (16–18). Data on maximum bilirubin levels were not obtained in this study, as blood samples were not collected after exposure to excessive stress or starving.

As already mentioned, GS is a contributing factor in hyperbilirubinemia development in several hereditary hematological conditions. Excessive production of unconjugated and monoconjugated bilirubin, together with chronic hemolysis, lead to jaundice development as well as gallstone formation. In our cohort, hematological causes of hyperbilirubinemia were excluded based on routine microscopic red blood cells examination. However, several authors report that the presence of hereditary spherocytosis can be masked by coexistence with GS (19–21). Therefore, when hereditary anemia is suspected more specific tests should be carried out to diagnose this condition.

In this study, we have identified three patients with allele (TA)$_8$, which is usually rarely present in Caucasians. The presence of the (TA)$_8$ allele was reported for the first time by Iolascon et al. in an Italian girl with GS (22). Thereafter, few studies have also described patients with this allele (23–25). Two of our patients with (TA)$_7$/(TA)$_8$ genotype had hyperbilirubinemia and were clinically diagnosed with GS which, surprisingly, did not differ from patients with the (TA)$_7$/(TA)$_7$ genotype. Considering the fact that the UGT1A1 promoter activity of (TA)$_8$ is the lowest one (7), these patients were expected to have the highest bilirubin levels. The same finding on similarity of the phenotype of (TA)$_7$/(TA)$_7$ and (TA)$_7$/(TA)$_8$ subjects was also reported by Ostanek et al. (25).

The subject with heterozygous genotype (TA)$_5$/(TA)$_8$ did not have hyperbilirubinemia, neither in the time of testing nor in the anamnestic data. Because only one patient with this genotype was identified in our
Study, we were not able to make any general conclusions, but we could hypothesize that one wild type (TA)_6 allele might be enough to ensure sufficient UGT1A1 activity and prevent hyperbilirubinemia, similar to the (TA)_5/(TA)_7 patients.

GS is a contributory factor in neonatal hyperbilirubinemia and breast milk jaundice in newborns (4). It was suggested that newborns with (TA)_5/(TA)_7 and (TA)_5/(TA)_6 genotypes are predisposed to developing prolonged neonatal jaundice (26). On the other hand, Maruo and colleagues described an association of missense UGT1A1 mutation resulting in substitution G71R, with severe breast milk jaundice in Japanese newborns (27). A specific compound in milk that inhibits glucuronidation has not been identified, but lower UGT1A1 activity in newborns with GS was suggested to be a trigger of jaundice development. Thus, to the best of our knowledge, data on an association between the (TA)_6 allele and breast milk jaundice has not been published to date. For this reason, our data on two patients with the (TA)_5/(TA)_7 genotype who developed breast milk jaundice with high bilirubin levels (107.2 and 65.9 μmol/L, respectively) after breast feeding are even more interesting.

Out of different TA repeats in the UGT1A1 promoter region, the (TA)_5/(TA)_7 genotype was found to be the most common in Croatian subjects suspected of having GS. This genotype was present in more than half of the subjects of our cohort, indicating the importance of UGT1A1 genotyping. Furthermore, several cases with five or eight TA repeats were identified. Because the appearance of these alleles was associated with GS or breast milk jaundice in all our subjects, we consider that, even though rarely found, these variants should be properly identified and methods for confirming (TA)_6 and (TA)_7 alleles should be accessible in laboratories performing UGT1A1 genotyping.

Acknowledgements

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References


Table 4  Demographic and clinical characteristics of subjects with (TA)_5 or (TA)_7 alleles.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Genotype</th>
<th>Gender</th>
<th>Age</th>
<th>Total bilirubin, μmol/L</th>
<th>Rifampicin test</th>
<th>Clinical phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(TA)_5/(TA)_6</td>
<td>F</td>
<td>40 y</td>
<td>11.9</td>
<td>Not performed</td>
<td>Normal (mother-family study)</td>
</tr>
<tr>
<td>2</td>
<td>(TA)_5/(TA)_6</td>
<td>M</td>
<td>1 mo</td>
<td>107.2</td>
<td>Not performed</td>
<td>Breast milk jaundice</td>
</tr>
<tr>
<td>3</td>
<td>(TA)_5/(TA)_7</td>
<td>F</td>
<td>2 mo</td>
<td>65.9</td>
<td>Not performed</td>
<td>Breast milk jaundice</td>
</tr>
<tr>
<td>4</td>
<td>(TA)_5/(TA)_6</td>
<td>M</td>
<td>13 y, 4 mo</td>
<td>27.4</td>
<td>Positive</td>
<td>Gilbert’s syndrome + myopathy</td>
</tr>
<tr>
<td>5</td>
<td>(TA)_5/(TA)_7</td>
<td>F</td>
<td>10 y, 11 mo</td>
<td>29.1</td>
<td>Positive</td>
<td>Gilbert’s syndrome + chronic hepatitis</td>
</tr>
<tr>
<td>6</td>
<td>(TA)_5/(TA)_7</td>
<td>M</td>
<td>10 y, 11 mo</td>
<td>22.0</td>
<td>Positive</td>
<td>Gilbert’s syndrome</td>
</tr>
<tr>
<td>7</td>
<td>(TA)_5/(TA)_7</td>
<td>F</td>
<td>12 y, 1 mo</td>
<td>35.1</td>
<td>Positive</td>
<td>Gilbert’s syndrome</td>
</tr>
</tbody>
</table>

M, male; F, female; y, years; mo, months.


