(TA)$_8$ allele in the UGT1A1 gene promoter of a Caucasian with Gilbert's syndrome

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ABSTRACT

Background and Objective. Gilbert's syndrome, a chronic non-hemolytic unconjugated hyperbilirubinemia, is caused by a reduction in the activity of hepatic bilirubin UDP-glucuronosyltransferase (UGT1A1). This reduction has been shown to be due to a polymorphism in the promoter region of the UGT1A1 gene. The presence of seven thymine adenine (TA) repeats reduces the efficiency of transcription of the UGT1A1 gene. To elucidate the genetic background of a patient affected by Gilbert's syndrome, we collected blood samples from family members for the analysis of the A(TA)$_n$TAA motif in the promoter region of the UGT1A1 gene.

Design and Methods. Analysis of the A(TA)$_n$TAA motif in the promoter region of the UGT1A1 gene was performed by PCR. Estimation of UGT1A1 promoter containing the variable (TA) repeats was performed by using a luciferase reporter system.

Results. Three different genotypes were identified due to the presence of (TA)$_6$, (TA)$_7$ and (TA)$_8$ repeats. The production of luciferase decreases in inverse relation to the number of repeats.

Interpretation and Conclusions. The (TA)$_7$ polymorphism, associated with Gilbert syndrome, is the only allele found up to now in white populations, while two other variants (TA)$_5$ and (TA)$_8$ have been identified in black populations. We describe here the first case of a subject affected by Gilbert's syndrome who is heterozygous for the (TA)$_8$ allele in the promoter region of the UGT1A1 gene. This polymorphism, as well as the (TA)$_7$ one, is associated with an increased level of bilirubin and a significant reduction of transcription activity of the UGT1A1 gene.
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G ilbert's syndrome is an inherited form of mild unconjugated hyperbilirubinemia associated with decreased bilirubin UDP-glucuronosyltransferase activity.[1] The disease affects 6-

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12% of individuals, whose serum bilirubin levels range between 52 and 86 mmol/L.[2,3] Bilirubin values fluctuate with time and may increase during intercurrent illness or fasting,[4] as well as during the first days of life, when the increased bilirubin levels are a consequence of shortened erythrocyte life span and a reduced bilirubin glucuronide conjugation capacity due to liver immaturity.

The recent identification of the UGT1 locus, which encodes for a family of UDP-glucuronosyltransferase isoforms (A-M), has provided the tools for the molecular study of Gilbert's syndrome.[5,6] The isoforms derive from a unique UGT1 gene containing four exons present in all transcripts, and at least another 13 differently spliced sequences. A single alternative exon is spliced to the four common exons and the resulting mRNA codes the UDP-glucuronosyltransferase 1 isoenzyme (UGT1A1). Treatment with drugs, such as phenobarbital, induces transcription of the UTG1A1 gene in human or hepatoma cell lines and reduces the serum bilirubin level in Gilbert's patients.[7] Although heterozygous missense mutations of the UGT1A1 gene have been identified in patients with Gilbert's syndrome, most cases are associated with a polymorphism in the promoter region,[8,9] where an unusual 'TATA' box exists in two different forms, A(TA)$_6$TAA and A(TA)$_7$TAA, due to six and seven TA repeats, respectively.[2,10] The presence of this expanded element reduces the efficiency of transcription of the UGT1A1 gene.[10]

The less frequent allele (TA)$_7$ is associated with increased bilirubin levels in normal people,[10] in individuals heterozygous for β-thalassemia,[11] in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency,[12] in jaundiced newborns affected by G6PD deficiency[13] and hereditary spherocytosis.[14] Recently, the (TA)$_7$ form has been found to be more common among people of African origin than among those of Caucasian ancestry.[15] The total serum bilirubin level in Africans is, however, lower than that in Europeans.[16] In addition, whereas the (TA)$_7$ polymorphism is the only allele found in white populations, another two variants, (TA)$_5$ and (TA)$_8$, have also been identified in a black population.[15] Consistent with previous data, the transcriptional activity of the UGT1A1 promoter decreases with the

progressive increase of the number of TA repeats.

We describe here the first *white* subject affected by Gilbert's syndrome found to be heterozygous for the $(TA)_8$ allele in the promoter region of the UGT1A1 gene. This polymorphism, as well as $(TA)_7$, is associated with an increased level of bilirubin. Transfection of constructs containing different variants of the UTG1A1 promoter resulted in a significant reduction of the transcription activity of $(TA)_7$ and $(TA)_8$ compared to that of the wild type $(TA)_6$ allele.

Design and Methods

Human subjects

Blood specimens were collected from an 8-year-old girl with Gilbert's syndrome, her parents, her 3 year old brother, and two relatives. The patient was born in the 40th week of gestation and weighed 3,400 g at birth. Jaundice appeared 3 days after birth, and the indirect bilirubin level was 60 μMol/L. Direct and indirect Coombs' tests were negative. All members of this family were born in Taranto (Apulia), Italy.

Determination of UGT1A promoter genotype

Genomic DNA was obtained by standard methods from peripheral leukocytes.[17] Analysis of the $A(TA)_nTAA$ motif, in the promoter region of the UGT1 gene was performed by PCR, according to Bosma *et al.*[10] followed by separation of the amplified products on 6% denaturing polyacrylamide gel.

Functional analysis of the UTG1A1 promoter

UTG1A1 promoter fragments, 319-323 bp in length depending on the number of TA repeats, were amplified using primers F (5′-TGTAGATCTTCCTCTCTGGTAA-CACT-3′) and R (5′-ATGAAGCTTTGCTCCTGCCA-GAGGTTC-3′) from human genomic DNA of individuals homozygous or heterozygous for six, seven, and eight TA. To facilitate subcloning of the PCR products in the reporter gene construct, oligonucleotides F and R were designed with a *Bgl*II and *Hind*III restriction enzyme site at their respective 5′ ends. PCR was performed on 50 μL, using a TaKaRa Ex Taq Kit (TaKaRa BIOMEDICALS), which contains a TaKaRa Ex Taq polymerase for high fidelity. Initial denaturation at 94°C lasted 10 min, and was followed by amplification for 30 cycles with denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. Products of expected sizes were double digested with *Bgl*II and *Hind*III, purified by Quiaex kit (Quiagen), and subcloned into pGL2-Basic vector (Promega). The integrity of the resulting plasmids, named p6TA, p7TA and p8TA, was confirmed by restriction mapping and sequencing analysis. Promoter activity was measured in a human colon carcinoma cell line (Caco-2, ATCC HTB37). The UGTA1A gene was expressed, as determined by RT-PCR, in these cells (data not shown), and the efficiency of transfection was relatively high. Cells were grown in MEMa medium supplemented with 10% of fetal bovine serum (GIBCO) at 37°C with 5% CO_2.

Exponentially growing cells were trypsinized, seeded at 2.5×10^5 cells, and incubated overnight prior to transfection. Transfection was carried out by calcium phosphate precipitation[18] using 3 μg of each of the following constructs: p6TA, p7TA, p8TA, pGL2-Basic, and pGL2-Control as a positive control, the luciferase gene being under the control of the SV40 promoter and enhancer. To monitor trasfection efficiency, plasmid pSV-β-Gal (Promega) was also co-transfected. Cells were treated with a reporter lysis buffer (Promega) and incubated at room temperature for 30 min before harvesting. The centrifuged cell extracts were assayed for both luciferase and β-galactoside activities according to Promega protocols. Luciferase activity was normalized to β-galactosidase activity. Normalized luciferase activities were expressed as ratios to those seen with pGL2-Basic vector. At least four independent experiments were performed for each construct and all measurements were determined in duplicate.

Results

To elucidate the genetic background of a patient affected by Gilbert's syndrome, we collected blood samples from each family member for the analysis of the $A(TA)_nTAA$ motif in the promoter region of the UGT1A1 gene. Three different genotypes were identified due to the presence of $(TA)_6$, $(TA)_7$, and $(TA)_8$ repeats (Figure 1). The proband and her mother were heterozygous for two alleleic variants, $(TA)_7$ and $(TA)_8$. The bilirubin levels in these subjects were con-

Figure 1. Family pedigree: II.2 and III.1 have Gilbert's syndrome.
6/6 genotype= A(TA)6TAA/A(TA)6TAA;
6/7 genotype= A(TA)6TAA/A(TA)7TAA;
6/8 genotype= A(TA)6TAA/A(TA)8TAA;
7/8 genotype=A(TA)7TAA/A(TA)8TAA.
Serum bilirubin levels (μM/L) of the subjects above.
Polyacrylamide gel electrophoresis of a PCR product containing UGT1A1 promoter region. Lane 1 heterozygous 6/8; lane 2 homozygous 6/6; lane 3 heterozygous 7/8; lane 4 heterozygous 6/7; lane 5 proband heterozygous 7/8; lane 6 heterozygous 6/8.
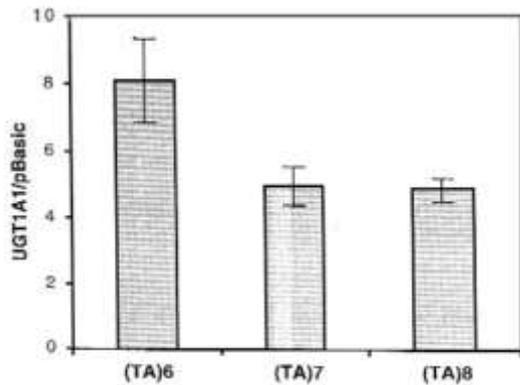
Figure 2. Estimation of the UGT1A1 promoters containing the variable (TA) repeats using a luciferase reporter system. Each construct was transiently transfected into Caco2 cells. The β-galactosidase reporter gene was cotransfected as an internal control. Luciferase activity was normalized to β-galactosidase activity. The results are normalized to the value of the transcriptional activity of the promoter less vector (pGL2-Basic) and are indicated as Fold/pBasic activity. Means values of four experiments in duplicate and their standard errors are shown.

sistent with those found in patients with Gilbert's syndrome (Figure 1). The genotypes of the father and the younger brother were 6/7 and 6/8, respectively, and those of two other relatives were 6/6 and 6/8. The serum bilirubin values in these four individuals were within the normal range (Figure 1).

To determine whether the number of repeats can modulate promoter activity of the UGT1A1 gene, fragments with six to eight TA repeats were subcloned upstream of the reporter luciferase gene. These constructs were tested by transient transfection of Caco-2 cells, a colon carcinoma cell line that expresses the UGTA1A gene (data not shown). The production of luciferase decreased in inverse relation to the number of repeats (Figure 2). The activity of the promoter containing $(TA)_7$ repeats was approximately 50% lower than that of the most common promoter with $(TA)_6$. The construct with $(TA)_8$ provided only a small difference in activity compared to the $(TA)_7$ promoter.

Discussion

In this paper we describe a family from the south of Italy with two individuals affected by Gilbert syndrome, heterozygous for two alleleic variants, $(TA)_7$ and $(TA)_8$ in the $A(TA)_nTAA$ motif in the promoter region of the UGT1A1 gene. As previously reported,[15] the number of TA repeats correlated with different serum bilirubin levels and different transcriptional activity of the respective promoters. In particular, $(TA)_7$ and $(TA)_8$ were associated with higher levels of serum bilirubin probably due to decreased transcription efficiency.

The TATAA element is the binding site for the tran-

scription factor IID and plays an important role in the initiation of transcription.[18,19] Variations of this sequence might, therefore, result in a reduction of the efficiency and accuracy of transcriptional activity.[18,20] Additional TA repeats might decrease the rigidity of the DNA near to the TATA box and alter the interaction of nuclear proteins with regulatory sequences.[21]

Among Europeans, the most common mutation associated with Gilbert's syndrome is insertion of an extra TA into the promoter region of the UGT1A1 gene. Mutations in the coding region of this gene cause the more severe Crigler-Najjar syndrome,[22] and heterozygotes for such mutations may manifest a dominant form of Gilbert's syndrome.

In Caucasian people, the $(TA)_7$ allele is extremely common, occurring with a frequency of 0.38, whereas the $(TA)_8$ allele has never been detected up to now.[15] The frequency of the $(TA)_7$ promoter is lowest in the Asian population and highest in the African population in which two other variants have been identified, $(TA)_5$ and $(TA)_8$, occurring with relative frequencies of 0.035 and 0.069, respectively.

Approximately 10,000 European patients with Gilbert's syndrome have been studied, but there have been no reports of the $(TA)_8$ allele. Our own group has studied the promoter region of the UGT1A1 gene of at least 600 subjects with hyperbilirubinemia and the family reported in this paper is the first and only to show the $(TA)_8$ variant. We can conclude that the $(TA)_8$ promoter repeat is an extremely rare mutation in Caucasians. This observation might suggest that the $(TA)_8$ allele found in our family, unlike other alleles present in the population from Southern Italy, such as HbS, G6PD and I/65 mutation in elliptocytosis, does not derive from African chromosomes but it is probably a recent spontaneous mutation.

Like microsatellites, repeated sequences are extremely unstable and may be lengthened or shortened by a variety of mechanisms, such as unequal crossing over in meiosis or slippage during DNA replication.[23] In conclusion, in our experience (more than 1 thousand chromosomes examined coming from the same area) no other cases of abnormal $(TA)_8$ repeat exist. Haplotype analysis of chromosomal region in proximity to the UTG1A1 gene will provide more evidence on the origin of the Italian $(TA)_8$ allele.

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