

results. Similarly, we observed no differences between heparin-plasma and serum samples analyzed on the Vitros system. The layered slide technology on the Vitros prevents precipitation penetrating through to the reaction layer. When we measured glucose, we observed no differences between the heparin-plasma or serum samples with the Rapidlab 865. The membrane on the glucose sensor also prevents precipitation from potentially causing reaction problems.

In conclusion, 2 samples with IgM- κ paraproteins produced interference with the Roche glucose and GGT methods when collected as lithium heparin-plasma samples. Automatic reruns gave the same error flags. The best way to obtain accurate glucose and GGT results for these patients was to use a serum sample or a different measurement system for glucose analysis.

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The Polymorphism c.-3279T>G in the Phenobarbital-Responsive Enhancer Module of the Bilirubin UDP-Glucuronosyltransferase Gene Is Associated with Gilbert Syndrome, Elísio Costa,^{1*} Emília Vieira,² and Rosário dos Santos² (¹ Escola Superior de Saúde do Instituto Politécnico de Bragança, Bragança, Portugal; ² Unidade de Genética Molecular do Instituto de Genética Médica Dr. Jacinto de Magalhães, Porto, Portugal; * address correspondence to this author at: Escola Superior de Saúde, Instituto Politécnico de Bragança, Avenida D. Afonso V, 5300-121 Bragança, Portugal; fax 351-273-327915, e-mail elisio.costa@ipb.pt)

Hepatic glucuronization of water-insoluble bilirubin is catalyzed by isoenzyme 1A1 of UDP-glucuronosyltrans-

ferase (UGT1A1), which is essential for efficient biliary excretion of bilirubin. Mild hyperbilirubinemia, usually <50 $\mu\text{mol/L}$, is associated with Gilbert syndrome (GS) and is thought to reflect a small reduction in UGT1A1 activity (1,2). The main cause of GS in all studied populations is a TA insertion in the repetitive TATA box of the gene promoter (3-6), which usually consists of 6 repeats. This (TA)₇ allele is extremely common, occurring with an estimated frequency of 38.7% in the white population and 16% in the Asian population (7). In persons with African ancestry, the number of alleles carrying 7 or more repeats is reported to be 49.5% (7). On the basis of the frequencies observed in Caucasians in several studies of smaller series, Beutler et al. (7) calculated a homozygosity frequency of 15%. This predicted value is higher than that in patients with a clinical diagnosis of GS, suggesting incomplete penetrance of the (TA)₇ allele. Moreover, the fact that some GS patients are neither homozygous for the (TA)₇ allele nor have other UGT1A1 genetic deficits points toward the existence of other inherited or acquired factors affecting bilirubin metabolism.

Phenobarbital treatment for hyperbilirubinemia increases UGT1A1 activity in the liver via a phenobarbital-responsive enhancer sequence with 3 potential nuclear receptor (NR) motifs separated by ~90 bases (8). From the 5' end, these motifs are named NR4, gtNR1, and NR3. Alterations in this region could be related to hyperbilirubinemia (8). A polymorphism (c.-3279T>G) located in the NR3 motif is associated with a decrease in transcriptional activity, to ~62% of wild type (9). This polymorphism was found in patients with GS and the (TA)₇ allele (9,10). More recently, a similar cooperative effect has been observed in cancer patients showing severe irinotecan toxicity (11).

The aim of the present study was to screen for the presence of alterations in the phenobarbital-responsive enhancer sequence of the UGT1A1 gene and to investigate a possible association of these alterations with GS.

We studied 80 unrelated Caucasian patients (53 males and 27 females) 14 to 45 years of age. The diagnosis of GS was based on standard criteria of mild unconjugated hyperbilirubinemia, normal liver function tests, and no overt signs of hemolysis. The mean (SD) total bilirubin concentration was 29.2 (10.07) $\mu\text{mol/L}$. Sixty-seven patients were homozygous for the (TA)₇ allele in the promoter region of the UGT1A1 gene, and 13 were heterozygous. Direct sequencing of the 5 exons and promoter region (c.-1568 to c.-1043) in these (TA)₇ heterozygotes revealed no additional alterations. We also studied 75 Caucasian individuals with no known history of jaundice (control group).

In all 80 GS patients, the phenobarbital-responsive enhancer sequence of the UGT1A1 gene (c.-3576 to c.-3209) was sequenced using the primers UGT1A1regF (5'-CTAGCCATTCTGGATCCCTTG-3') and UGT1A1regR (5'-TTTTGAGATCTGAGTTCTCTTCACCTC-3'). PCR was performed in a 50- μL volume containing 25 μL of PCR Master Mix (Promega), 22 μL of water, 1 μL of each primer (50 pmol/ μL), and 1 μL (250 ng) of genomic DNA.

Amplification was performed in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems) with an initial denaturation step of 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. The amplicons were isolated by electrophoresis on a 2% agarose gel and then purified by use of the spin-X[®] (Corning Incorporated). The nucleotide sequence was determined, with use of the respective forward and reverse primers, in independent sequencing reactions with the Dye Terminator Cycle Sequencing Kit (Perkin-Elmer) and subsequently resolved on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

In the control group, single-strand conformational analysis was performed for carrier detection of the c.-3279T>G polymorphism within the phenobarbital-responsive enhancer sequence. Amplicons were electrophoresed at room temperature on 0.5× MDE gels (BMA Products) and visualized by conventional silver staining methods.

The control group was also screened for the (TA)₇ allele in the promoter region of the *UGT1A1* gene. This TATA box region was analyzed by PCR amplification using the primers described by Bancroft et al. (12), with the introduction a fluorochrome label in the reverse primer for subsequent analysis by automated capillary electrophoresis. PCR was performed in a 50-μL volume containing 25 μL of PCR Master Mix (Promega), 22 μL of water, 1 μL of each primer (50 pmol/μL), and 1 μL (250 ng) of genomic DNA. PCR amplification was performed in a GeneAmp PCR System 9600 thermal cycler with an initial denaturation step of 95 °C for 10 min, followed by 35 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, with final extension at 72 °C for 30 min. After PCR, 1-μL portions of the products were separated by automated capillary electrophoresis and analyzed by GeneScan (Ver. 3) software (Applied Biosystems), along with the molecular weight marker ROXTM-500 size standard (Applied Biosystems).

Direct sequencing of the phenobarbital-responsive enhancer sequence in the GS patients revealed only the presence of the c.-3279T>G polymorphism, which was found in homozygosity in 74 (92.5%) and heterozygosity

in 6 (7.5%) of the patients (Table 1). The frequency of this polymorphism was significantly higher in the GS group than in the control group (96.25% vs 41.33%; $\chi^2 = 110.5$; $P < 0.001$). The great majority of GS patients and control group members homozygous for the (TA)₇ allele were also homozygous for the c.-3279T>G polymorphism (95.83%). In individuals heterozygous for the (TA)₇ allele, the presence of homozygosity for the c.-3279T>G polymorphism was significantly higher in the GS group (69%) than in the control group (28%; $\chi^2 = 6.461$; $P = 0.011$).

In the control group, we found a compound heterozygote for the (TA)₅ and (TA)₇ alleles associated with homozygosity for the c.-3279T>G polymorphism.

We found no statistically significant difference among bilirubin concentrations in the different patient haplotypes. This analysis, however, was limited by fewer numbers of cases in 3 of the 4 genotype combinations. Further comparisons and statistical analyses were also inhibited because no bilirubin concentrations were known for the anonymized control group.

Homozygosity for the (TA)₇ allele was initially considered to be the sole cause of GS in Caucasians (3–6); however, it has become increasingly evident that the genotype/phenotype relationship is not linear (6, 7, 10, 13). On the one hand, the bilirubin concentrations in such patients are very heterogeneous, whereas on the other hand, some people without GS are also found to be homozygous for this allele. For this reason, many authors search for other factors that could influence phenotypic expression.

In the present study, the c.-3279T>G polymorphism was a common finding in both GS and control individuals. Its much higher frequency in the GS group suggests, however, that it may be a significant contributor.

The (TA)₇ allele decreases the transcriptional activity of the *UGT1A1* gene to only 60%–80% of normal (4, 7), and the c.-3279T>G polymorphism decreases it to ~62% of normal (9). The association of these 2 variants could thus lower the transcriptional activity of the *UGT1A1* gene to as little to as 37% of normal. Almost all of our GS patients who were homozygous for the (TA)₇ allele were also homozygous for the c.-3279T>G polymorphism, suggest-

Table 1. Distribution of the c.-3279T>G polymorphism and (TA)₇ insertion in control and GS groups.

Group	TATA box [A(TA) ₇ TAA]	c.-3279T>G		
		Wild type	Heterozygous	Homozygous
GS patients (n = 80)	Wild type (n = 0), n	0	0	0
	Heterozygous (n = 13)			
	n	0	4	9
	Mean (SD) bilirubin, μmol/L		35 (13.89)	23.72 (6.88)
	Homozygous (n = 67)			
	n	0	2	65
Controls (n = 75) ^a	Mean (SD) bilirubin, μmol/L		28.4 (11.17)	26.7 (6.88)
	Wild type (n = 40), n	24	15	1
	Heterozygous (n = 29), n	3	18	8
	Homozygous (n = 5), n	0	1	4

^a One patient was heterozygous for the (TA)₅ and (TA)₇ alleles.

ing a linkage between these 2 markers. Indeed, linkage disequilibrium between these 2 markers was recently demonstrated in a group of 133 healthy Caucasian individuals (14).

We also found homozygosity for the c.-3279T>G polymorphism in a large number (~70%) of our GS patients who were heterozygous for the (TA)₇ allele. This genotype could be sufficient to justify the clinical picture found in these patients, as could compound heterozygosity for (TA)₇ and c.-3279T>G.

In summary, these results suggest that the c.-3279T>G polymorphism of the *UGT1A1* gene could be an additional, if only cumulative, risk factor for the development of GS, thus justifying the inclusion of this polymorphism in routine molecular screening protocols.

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