supplying equipment (Behring Nephelometer II) and reagents for nephelometric measurement of urinary albumin. We also thank J. van der Wal-Hanewald (laboratory assistant) for concise and meticulous work.

References


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Use of Fully Denaturing HPLC for UGT1A1 Genotyping in Gilbert Syndrome, James R. Harraway * and Peter M. George (Molecular Pathology Laboratory, Canterbury Health Laboratories, Christchurch, New Zealand; * address correspondence to this author at: Molecular Pathology Laboratory, c/o Canterbury Health Laboratories, Christchurch, NA 8001, New Zealand; fax 64-3-3640545, e-mail jharraway@rcpa.edu.au)

Gilbert syndrome is an inborn error of bilirubin conjugation caused by mutations in the conjugating enzyme uridine diphosphate glucuronosyltransferase (UGT1A1) (1). UGT1A1 activity is moderately reduced in Gilbert syndrome, causing low-grade nonhemolytic unconjugated hyperbilirubinemia without other biochemical or clinical features of hepatic or hematologic pathology. Bilirubin concentrations typically fluctuate over time and tend to be increased in response to metabolic stress such as intercurrent illness and fasting (2).

The clinical importance of Gilbert syndrome derives from its high population prevalence. The causative mutation in Caucasians is almost exclusively a dinucleotide insertion in the TATA box of the UGT1A1 promoter. The most common UGT1A1 promoter contains the sequence (TA)2(TAA); insertion of an extra TA reduces expression of the UGT1A1 gene to 20%–30% of reference values (3). The frequency of the (TA)2(TAA) allele (also known as UGT1A1*28 in the Caucasian population) is ~0.35; therefore, nearly all Caucasians with Gilbert syndrome are homozygous for this allele (4). UGT1A1*28 is less frequent in Asians. A significant proportion of Asians with Gilbert
syndrome have UGT1A1*28 as one mutant allele, but other substitutions that diminish UGT1A1 activity are more common, including p.G71R and p.P229Q (5). Indeed, given the frequency of UGT1A1*28, 10%–15% of Caucasians are homozygous for this allele; however, the prevalence of clinical Gilbert syndrome has been estimated at 5%. The difference between these 2 values may reflect an ascertainment bias, relatively low penetrance of UGT1A1*28 homozygosity, or a combination of both (1, 3).

A variety of assays adaptable for clinical use can detect the UGT1A1*28 allele, such as polycrylamide gel electrophoresis (PAGE), direct sequencing, fluorescence resonance energy transfer with melting curve analysis, pyrosequencing, and denaturing HPLC (dHPLC); reviewed in Ref. (6)]. We used dHPLC to separate fragments based on size rather than heteroduplex melting characteristics as has been described previously (7). Our approach requires only one PCR reaction with unlabeled primers and uses lower-cost reagents than direct sequencing or fluorescence resonance energy transfer. Sample analysis takes ~10 min; therefore, operating times are shorter and labor costs lower than with direct sequencing or gel-based size separation.

A drawback of this technique is that samples cannot be processed in parallel, as can be done on a polycrylamide gel or automated sequencer. Another drawback of dHPLC (7) is that partially denaturing conditions are used to detect heteroduplex formation by a change in peak retention pattern. Thus, samples from heterozygous (TA)_n/(TA)_7 individuals can be detected, but there is no difference in dHPLC pattern between homozygous (TA)_n and (TA)_7 individuals. This problem can be solved (7) by admixing PCR products from a known (TA)_n individual with the sample PCR products and rerunning the analysis, but this step greatly increases the cost and time per sample. Our method separates PCR products based on molecular weight only and avoids the need for repeat analysis while retaining the advantages of dHPLC.

Samples from 60 patients with hyperlipidemia were analyzed for UGT1A1 genotype and for bilirubin concentration, because high bilirubin may be cardioprotective. DNA was extracted from EDTA-peripheral blood by use of a TECAN Genesis liquid handling platform and the AGOWA Mag Maxi DNA Isolation Kit (Agowa), according to the manufacturer’s instructions.

PCR primers were designed from the GenBank reference sequence for UGT1A1 to amplify a small (59-bp) amplicon containing the UGT1A1 promoter TATA box. Primer sequences were 5′-ACT TGG TGT ATC GAT TGG TTT TTG-3′ (forward) and 5′-GCC TTT GCT CCT GCC AGA GG-3′ (reverse). Amplification was carried out on an MG thermocycler (Eppendorf). Optimized PCR conditions were as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 65 °C for 30 s. Reaction mixtures included 2.5 μL of Optimase 10× PCR reaction buffer + magnesium (Transgenomic), 2.5 μL of 2 mM deoxynucleotide triphosphates, 1 μL each of 10 μM forward and reverse primer, 0.2 μL of Optimase polymerase (Transgenomic), 100 ng of extracted DNA, and water to a final volume of 25 μL.

PCR amplicons were separated under fully denaturing conditions on a WAVE dHPLC (Transgenomic). The stationary phase used was a column of 2-μm nonporous alkylated polystyrene-divinylbenzene, with a mobile phase consisting of a mixture of buffers A (0.1 mol/L triethylammonium acetate, pH 7) and B (250 mL/L acetonitrile in 0.1 mol/L triethylammonium acetate, pH 7). The NAVIGATOR software supplied with the WAVE was used to program the desired gradient of buffer B. Buffer concentrations at the start of the program were 31% B, with a gradient over the 7-min run time to 38% B, and the flow rate was 0.9 mL/min. Run temperature was constant at 80 °C, and eluted DNA fragments were detected at 260 nm.

Confirmatory DNA sequencing was carried out on an ABI Prism 3100 Avant automated sequencer using the ABI PRISM Dye Terminator Cycle Sequencing Kit, Ver. 3.0 (Applied Biosystems). PCR amplicons were purified with the Amicon MultiScreen-PCR 96-Well Filtration System (MANU030; Amicon) according to manufacturer instructions, and sequenced with both forward and reverse primers. Additional samples were retested by electrophoresis on 12% polyacrylamide gel (1.25 h at 150 V) to further confirm the dHPLC genotype.

Samples eluted from the column with peaks at a mean (SD) of 5.17 (0.05) min for the 6-allele and 5.72 (0.05) min for the 7-allele samples. Although there was slight sample-to-sample variation in elution time, all samples could clearly be called as 6/6, 6/7, or 7/7. Because of the possibility of variation of overall elution times with different columns and buffers, internal controls of known genotype were run with each batch of samples.

Because of the TA repeat nature of the target amplicon, some “stutter” bands were seen, e.g., a shadow band in the 6 position in a 7/7 individual. These stutter bands were clearly much smaller in amplitude than the main band, and there was a clear distinction between a 6/7 and a 7/7 pattern, as shown in Fig. 1.
Sequencing (10 samples) and PAGE (10 additional samples) confirmed the genotypes determined by dHPLC analysis. Fasting plasma bilirubin concentrations were obtained for each of the patients tested (Table 1). Allele frequencies in this population were 0.66 for the 6 allele and 0.34 for the 7 allele, concordant with previous values determined in Caucasian populations.

Using a heteroscedastic 2-tailed t-test, we determined that the combined bilirubin means of the 6/6 and 6/7 samples were significantly different from those of the 7/7 group (P = 0.005). Means of both the 6/6 and 6/7 groups alone were also significantly different from those of the 7/7 group (P = 0.004 and 0.006, respectively). There was no significant difference in mean bilirubin between the 6/6 and 6/7 groups (P = 0.46).

Our assay uses HPLC to separate PCR amplicons by size without relying on differences in denaturation, enabling each sample to be definitively genotyped within a single reaction more easily than with the only other published dHPLC method. A recent report described the use of pyrosequencing for detection of the UGT1A1 genotype. Pyrosequencing is similar in efficiency to the method described here, with rapid throughput and low cost per sample. As with all assays, the choice of method will partly depend on the platform available in the laboratory.

Our method was shown to be 100% concordant with a DNA sequencing and a PAGE method, and the genotypes obtained were in concordance with plasma bilirubin concentrations for each patient. The main disadvantage of this assay is the lack of parallel processing ability; at a certain threshold, a gel-based separation method such as PAGE might be more efficient. With our method, however, 96 samples can be processed overnight without operator intervention.

The initial step in investigation of jaundice should be to determine the conjugated bilirubin fraction; if this is increased, then hepatobiliary pathology should be further investigated. Predominantly unconjugated hyperbilirubinemia may be caused by Gilbert syndrome, increased bilirubin load attributable to hemolysis, or hepatic disease. Because the Gilbert syndrome genotype is common and is not 100% penetrant, homozygosity for the UGT1A1*28 allele in the context of jaundice would reduce the posttest probability of other causes but would not rule them out.

The assay should therefore be used only after exclusion of other causes of hyperbilirubinemia, and it will help to confirm a presumptive diagnosis of Gilbert syndrome. Demonstration of a (TA)6/(TA)6 or (TA)6/(TA)7 genotype in a Caucasian patient with jaundice would make Gilbert syndrome highly unlikely and indicate the need for further investigation.

Our assay provides rapid and low-cost genotyping of the UGT1A1*28 allele, which is responsible for the majority of cases of Gilbert syndrome in the Caucasian population. There is emerging evidence that UGT1A1 is a pharmacogenetic marker for drugs such as irinotecan and that it may be useful in assessing neonatal jaundice, suggesting future applications of this assay.

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References

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Table 1. Bilirubin concentrations for the 3 UGT1A1 genotype groups determined by dHPLC.

<table>
<thead>
<tr>
<th>UGT1A1 genotype</th>
<th>Mean (SD)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>6/6 (n = 28)*</td>
<td>8.8 (2.7)</td>
<td>4–14</td>
</tr>
<tr>
<td>6/7 (n = 23)</td>
<td>9.5 (3.5)</td>
<td>4–16</td>
</tr>
<tr>
<td>7/7 (n = 9)</td>
<td>20.1 (8.5)</td>
<td>11–39</td>
</tr>
</tbody>
</table>

* Number of patients with each genotype.