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Rapid molecular diagnosis of the Gilbert's syndrome-associated exon 1 mutation within the *UGT1A1* gene

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ABSTRACT. Gilbert's syndrome is suspected in patients with unconjugated hyperbilirubinemia caused by decreased activity of the *UDP-glucuronosyltransferase 1A1* (*UGT1A1*) gene in the absence of abnormal liver function and hemolysis. The major genetic variants underlying Gilbert's syndrome are TATA-box repeats of the promoter region and exon 1 G211A of the coding region, particularly in Asians. The efficacy of DNA melting curve analysis, however, has not been established for the G211A mutation. For rapid and accurate molecular diagnosis of Gilbert's syndrome, DNA melting curve analysis was evaluated for its genotyping capability not only for TATA-box

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repeats of the *UGT1A1* promoter, but also for G211A of *UGT1A1* exon 1. TA repeats within the TATA-box sequence and the exon 1 G211A mutation of the *UGT1A1* gene were analyzed by DNA melting curve analysis. To evaluate the assay reliability, direct sequencing or polyacrylamide gel electrophoresis was used as a comparative method. All homozygous and heterozygous polymorphisms of $A(TA)_7TAA$ within the TATA-box allele and of exon 1 G211A mutants of the *UGT1A1* gene were successfully identified with DNA melting curve analysis. DNA melting curve analysis is, therefore, an effective molecular method for the rapid diagnosis of Gilbert's syndrome, as it detects not only TATA-box polymorphisms but also the exon 1 G211A mutation located within the *UGT1A1* gene.

Key words: G211A mutation; *UDP-glucuronosyltransferase 1A1*; Gilbert's syndrome;

INTRODUCTION

Gilbert's syndrome is the most commonly inherited disorder of bilirubin metabolism, affecting 3-12% of the general population, and presents as mild unconjugated hyperbilirubinemia of around 1-5 mg/dL that becomes clinically apparent only during fasting, physical exercise, stress, intercurrent infections, or menstruation (Bosma et al., 1995; Monaghan et al., 1996; Borlak et al., 2000; Ruiz-Argüelles et al, 2005; Costa et al., 2006). The genetic variants of the *uridine 5'-diphosphate glucuronosyltransferase 1A1* (*UGT1A1*) gene, specifically in the TATA-box of the promoter region, might reduce the transcription activity of the gene and UGT1A1 enzyme concentration, to ultimately affect conjugation (hepatic glucuronidation) capacity, which results in Gilbert's syndrome (Bosma et al., 1995; Monaghan et al., 1996; Raijmakers et al., 2000; Hsieh et al., 2007). Affected subjects may experience an impairment in drug elimination as well as severe adverse effects from drug metabolites (e.g., acetaminophen, irinotecan, SN-38, indinavir) (de Morais et al., 1992; Ando et al., 1998, 2000; Burchell et al., 2000). Since the Food and Drug Administration has already recommended a reduced initial dose of the anti-cancer agent irinotecan for patients with Gilbert's syndrome, *UGT1A1* genotype analysis is critical for the prediction and prevention of adverse drug effects in these individuals (O'Dwyer and Catalano, 2006).

The most common *UGT1A1* genotype responsible for Gilbert's syndrome is the homozygous polymorphism $A(TA)_7TAA$ in the promoter region of the gene, that leads to a 70-80% reduction in glucuronidation activity (Bosma et al, 1995; Monaghan et al., 1996; Beutler et al., 1998; Biondi et al., 1999; Kadakol et al., 2000; Farheen et al., 2006), while the mutation at nucleotide 211 (G211A) that causes arginine to replace glycine at position 71 in the coding region of the *UGT1A1* gene is responsible for approximately 20% of Gilbert's syndrome cases in Asian subjects (Iolascon et al., 1999; Hsieh et al., 2001). Several approaches have been described for detection of the TATA-box polymorphism: a) direct sequencing, b) denaturing high-performance liquid chromatography, c) polymerase

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chain reaction (PCR) and polyacrylamide gel electrophoresis, d) high-resolution melting curve analysis (HRM), and e) DNA melting curve analysis (Bosma et al, 1995; Monaghan et al., 1996; Sampietro et al., 1998; Marziliano et al., 2000; Harraway and George, 2005). Likewise, a number of methods have been used to identify the G211A mutation: a) direct sequencing, b) PCR-restriction fragment length polymorphism (RFLP), and c) PCR with Taqman and the minor groove binder (MGB) SNP assay (Maruo et al., 1999; Huang et al., 2000; Wong et al., 2007). These techniques differ with respect to thoroughness, time consumption, sensitivity and specificity, as well as cost, and the need for special instruments. None of the mechanisms listed above, however, has been evaluated both for the TATA-box polymorphism and for the G211A mutation simultaneously.

Although, the TATA-box repeat polymorphism has been previously examined by DNA melting curve analysis in Caucasians (Borlak et al., 2000), the efficacy of this method for determining both the TATA-box sequence and presence of the G211A mutation within the *UGT1A1* gene is still unknown for Asian subjects. In the present study, we evaluated DNA melting curve analysis as a rapid and accurate molecular diagnostic test of Gilbert's syndrome, that not only examines the promoter region of the *UGT1A1* gene but also exon 1.

MATERIAL AND METHODS

Patient samples

From January 2007 to December 2009, one hundred patients who presented as unconjugated hyper- bilirubinemia with normal liver profile visited our outpatient Department of Gastroenterology and agreed to participate in the study. A written informed consent and a detailed medical history from participants were obtained by physicians. To evaluate the genotyping reliability of DNA melting curve analysis for the $A(TA)_nTAA$ motif of the *UGT1A1* promoter and G211A of *UGT1A1* exon 1, genomic DNA was analyzed using this method. The genotypes of the TATA-box were confirmed with direct sequencing and/or polyacrylamide gel electrophoresis, while those of exon 1 were confirmed with direct sequencing only. The study protocol was approved by the Institutional Review Board of Tri-Service General Hospital, Taiwan.

Isolation of human genomic DNA

Genomic DNA from patients was extracted with a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer protocol. Briefly, human whole blood was lysed with cell lysis solution and then incubated for 10 min at room temperature. After centrifugation, nucleus lysis solution and protein precipitation solutions were added to the pellet. After additional centrifugation at 16,000 *g* for 3 min at room temperature, the supernatant was transferred to a new tube containing isopropanol. After a final centrifugation step, the pellet was washed with 70% ethanol. Once the ethanol was aspirated, genomic DNA was resuspended in DNA rehydration solution.

Sequence analysis of the TATA-box and exon 1 of the *UGT1A1* gene

A 98- or 100-bp fragment encompassing the TATA-box element of *UGT1A1* was amplified by PCR from each genomic DNA sample using a sense primer (5'-GTCACGTGA CACAGTCAAAC-3') and an antisense primer (5'-TTTGCTCCTGCCAGAGGTT-3'). Exon 1 of the *UGT1A1* gene was also amplified by PCR using a sense primer (5'-AGGAGCAAAGG CGCCATGGC-3') and an antisense primer (5'-CTGGGATAGTGGATTTTGGT-3'). The sequences of the resultant PCR-amplified fragments were determined with DNA sequencing. Thermal cycler conditions were as follows: 94°C for 2 min, and then 25 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 1 min.

Electrophoretic analysis of the TATA-box of the *UGT1A1* gene

The TATA-box element of the *UGT1A1* gene of each collected genomic DNA sample was PCR-amplified as described above. The resultant fragments were analyzed on a 12% denaturing polyacrylamide gel that was first stained with ethidium bromide and subsequently exposed to UV light.

DNA melting curve analysis of the TATA-box and exon 1 of the *UGT1A1* gene

Fluorogenic adjacent hybridization probes were designed according to Roche Diagnostics recommended guidelines. The PCR primer pairs used for TATA-box analysis were previously described (Monaghan et al., 1996; Borlak et al., 2000). Probes were designed so that their melting temperatures were marginally higher than the melting temperatures of the primers. The anchor probe was labeled with fluorescein, while the sensor probe was labeled with Light Cycler Red 640 (LC-Red 640). The probes recognized adjacent sequences, with the shorter probe binding directly over the mutation site, but were separated by bases. Fluorescein was used as the donor fluorophore that, during PCR, blocked extension from the other probe. LC-Red 640 was used as the acceptor of the fluorescence resonance energy transfer (FRET) process, with a phosphorylated 3'-end to block extension. All probes were synthesized by TIB MOLBIOL (Berlin, Germany). The greater stability of the longer anchor probe enabled a loss in fluorescence when the shorter probe melted off the template. PCR was performed with primers for the TATA-box (primer A: 5'-AATGAACTCCCTGCTACCTT-3', primer B: 5'-CC ACTGGGATCAACAGTATCT-3') and for exon 1 (71 F: 5'-TAAGTAGGAGAGGGCGAAC C-3', 71 S: 5'-CATGCTGGGAAGATACTGTTGA-3', 71 A: 5'-CCTCCCTTTGGAATGGC-3', 71 R: 5'-CGAGACTAACAAAAGACTCTTTAC-3') with standard parameters according to the manufacturer protocol. PCRs included an anchor (5'-CTTTGCTCCTGCCAGAGGTTCG CCCT-FL-3' for the TATA-box, 5'-640-GTACAACGAGGCGTCAGGTGCTp-3' for exon 1) and sensor (5'-640-CCTACTTATATATATATATATATGGCAAAAACCp-3' for the TATA-box, 5'-GTGTAAAATGCTCCGTCTCTGA-FL-3' for exon 1) hybridization probe, 100 ng DNA, 4 mM $MgCl_2$, and 2 µL Light Cycler DNA master hybridization mix (Roche Applied Science, Mannheim, Germany) in a reaction mixture with a total volume of 20 µL. The reaction started with 95°C for 2 min, followed by 45 cycles of denaturation. PCR product analysis was performed by examining DNA melting curves in glass capillaries. DNA was denatured at 95°C

for 20 s, and maximal fluorescence was acquired by holding the reaction at 40°C for 20 s. Melting curve data were generated by subsequently slowly heating the DNA to 85°C with a ramp rate of 0.2°C/s, with data collection occurring continuously during that time. When the shorter probe melted off the template, FRET ceased. Fluorescence was converted to melting peaks with the Lightcycler Software 4.05.415 (Roche Applied Science) that plotted the negative derivative of fluorescence with respect to temperature.

RESULTS

DNA melting curve analysis is a rapid diagnostic method of genotyping for the TATA-box sequence within the *UGT1A1* promoter

To evaluate the genotyping reliability of DNA melting curve analysis on mutations associated with Gilbert's syndrome in Asians, genomic DNA from 71 subjects was analyzed. The *UGT1A1* genotypes of the promoter region were determined by direct sequencing, and found to be either homozygous wild-type $A(TA)_6TAA/A(TA)_6TAA$, heterozygous $A(TA)_6TAA/A(TA)_7TAA$, or homozygous $A(TA)_7TAA/A(TA)_7TAA$ mutant alleles (Figure 1A). Although polyacrylamide gel electrophoresis was sensitive and effective in identifying heterozygous $A(TA)_6TAA/A(TA)_7TAA$ alleles (Figure 1B, lane 2), homozygous patterns were ambiguous at times when pertaining to DNA fragments containing 98 bp $A(TA)_6TAA$ alleles instead of 100 bp $A(TA)_7TAA$ alleles (Figure 1B). The results of the DNA melting curve analysis revealed that $A(TA)_6TAA$ alleles resulted in a sharp melting peak at 55°C, whereas, the sharp melting peak was at 59°C for $A(TA)_7TAA$ alleles (Figure 1C). The TA insertion, therefore, resulted in a melting temperature shift of around 4°C, which allowed for easy detection of both the homozygous $A(TA)_6TAA$ alleles and the $A(TA)_7TAA$ alleles (Figure 1C). The genotyping sensitivity and specificity of DNA melting curve analysis were 1.00, compared with genotypes determined by direct sequencing and/or polyacrylamide gel electrophoresis (Table 1).

DNA melting curve analysis is a reliable molecular method of genotyping for the G211A mutation in *UGT1A1* exon 1

To determine the genotyping efficacy and reliability of DNA melting curve analysis on the G211A mutation of *UGT1A1* exon 1 in Asians specifically, we examined genomic DNA from 30 subjects. The *UGT1A1* exon 1-coding region genotypes were determined by direct sequencing, and found to be homozygous wild-type/wild-type, heterozygous wild-type/G211A, or homozygous G211A/G211A mutant alleles (Figure 2A). The results of the DNA melting curve analysis revealed that the homozygous wild-type alleles resulted in a sharp melting peak at 63°C, whereas, the sharp melting peak was at 55°C for the homozygous G211A mutant alleles (Figure 2B). The presence of G211A, therefore, resulted in a melting temperature shift of around 8°C, which allowed for easy identification of homozygous wild-type, heterozygous, and homozygous missense mutations in *UGT1A1* exon 1. The genotyping sensitivity and specificity of DNA melting curve analysis were 1.00, compared with genotypes determined by direct sequencing (Table 2).

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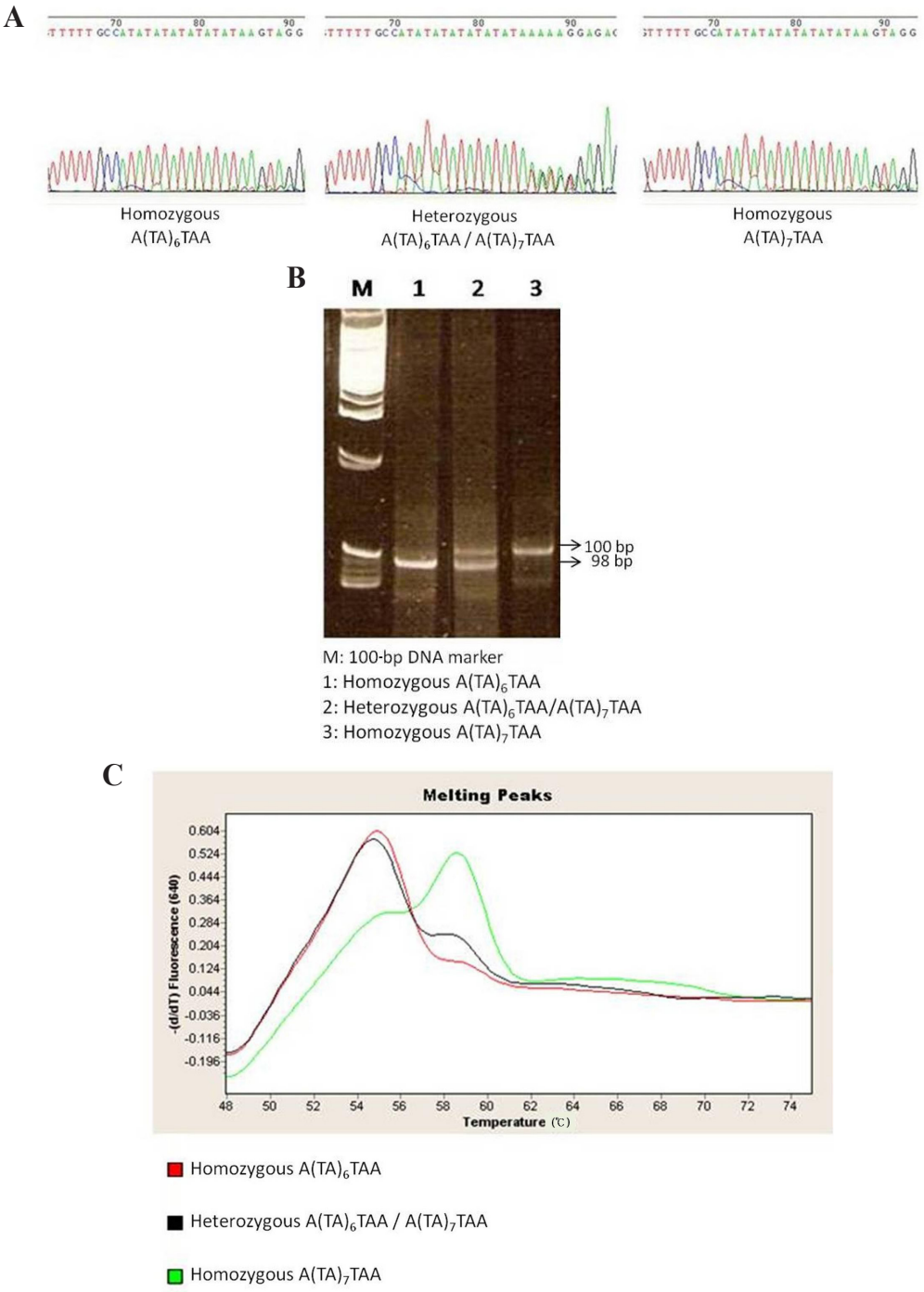

Figure 1. Genetic polymorphisms of the TATA-box promoter of the *UGT1A1* gene were identified by **A.** direct sequencing, **B.** polyacrylamide gel electrophoresis, and **C.** DNA melting curve analysis.

Table 1. Results of DNA melting curve analysis were compared to those of direct sequencing and/or polyacrylamide gel electrophoresis.

Melting curve analysis	*UGT1A1* TATA-box genotype		
	Homozygous $A(TA)_6TAA$	Heterozygous $A(TA)_6TAA/A(TA)_7TAA$	Homozygous $A(TA)_7TAA$
Positive	34	20	17
Negative	0	0	0

The sensitivity and specificity of DNA melting curve analysis were 1.00 and 1.00, respectively. Sensitivity: (20 + 17) / (20 + 17 + 0 + 0) = 1.00; specificity: 34 / (34 + 0) = 1.00.

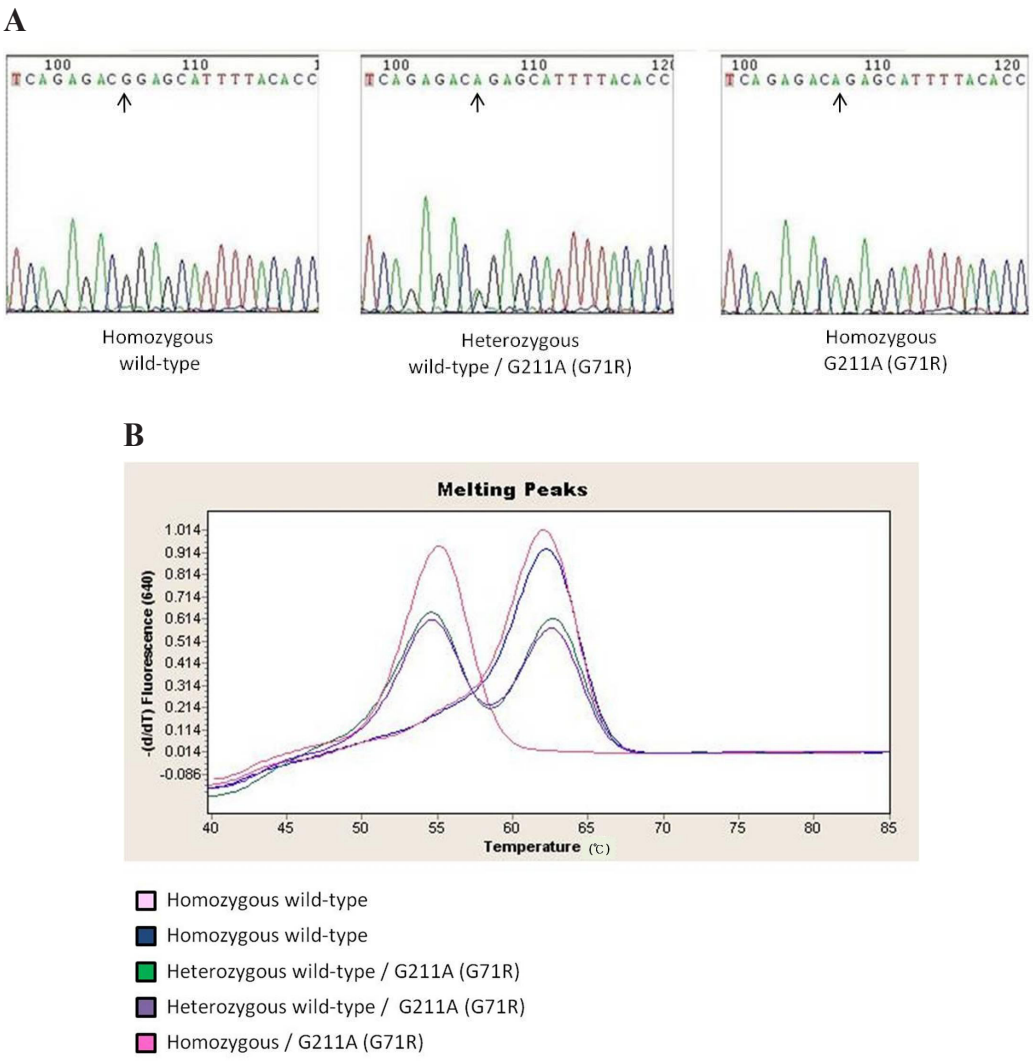

Figure 2. Genotypes of exon 1 G211A mutation were identified by **A.** direct sequencing and **B.** DNA melting curve analysis.

Table 2. Results of DNA melting curve analysis were compared to those of direct sequencing.

Melting curve analysis	*UGT1A1* exon 1		
	Homozygous wild-type	Heterozygous wild-type/G211A	Homozygous G211A
Positive	22	6	2
Negative	0	0	0

The sensitivity and specificity of DNA melting curve analysis were 1.00 and 1.00, respectively. Sensitivity: (6 + 2) / (6 + 2 + 0 + 0) = 1.00; specificity: 22 / (22 + 0) = 1.00.

DISCUSSION

According to the present study, we found that DNA melting curve analysis successfully detected all homozygous and heterozygous polymorphisms of $A(TA)_7TAA$ within the TATA-box allele and of exon 1 G211A mutants of the *UGT1A1* gene. Our TATA-box genotyping results were consistent with those of previous reports that used DNA melting curve analysis for diagnosis of Gilbert's syndrome (Borlak et al., 2000). As with direct sequencing, DNA melting curve analysis with the FRET assay provided an accuracy of 100% for the TATA-box polymorphism, which depended on the difference in melting temperature values between subject samples. Although DNA sequencing is clinically the gold standard diagnostic test of Gilbert's syndrome, it is limited by an ambiguous heterogeneous pattern, low throughput, high time consumption, and high cost. Polyacrylamide gel electrophoresis on the other hand is limited also by low throughout and time consumption, but additionally poorly differentiates between the homozygous wild-type $A(TA)_6TAA$ and the mutant $A(TA)_7TAA$ allele because there is only a 2-bp difference between them. HRM, the newest form of melting curve analysis, includes a special saturation dye and has the advantages of simplicity, high speed, low cost (about US$7.7 per reaction), and high sensitivity and specificity with resultant high-resolution images (Reed et al., 2007; Vossen et al., 2009; Minucci et al., 2010). Melting curve analysis with FRET probes is also simple, fast, inexpensive (approximately US$7.0 per reaction), but, furthermore, there is no need to purchase an additional special chemical buffer or instrumentation for it in an established laboratory. Melting curve analysis, therefore, is a reliable and cost-effective method for detection of the TATA-box polymorphism.

As for the TATA-box element, the following consideration is more relevant for the genetic mutation of the G211A allele. According to a previous study by Hsieh et al. (2001), 20% of Asians with Gilbert's syndrome risk being undiagnosed if only the polymorphism of the TATA-box promoter is searched for. There are, therefore, two points that require more attention when considering Asians as opposed to Caucasians: whether the G211A mutation exists or not and whether the methodology simultaneously screens for the TATA-box polymorphism and G211A mutants. Our data suggested that DNA melting curve analysis with FRET probes, equivalent to direct sequencing in its accuracy, could explicitly identify the G211A mutation and, thus, would be greatly reliable for the diagnosis of Gilbert's syndrome in Asian individuals. To the best of our knowledge, this is the first report that demonstrates the efficacy of DNA melting curve analysis in determining the genotype of G211A within *UGT1A1* exon 1 alongside the TATA-box polymorphism.

In addition to direct sequencing, PCR-RFLP and PCR with the Taqman MGB SNP assay have also been used to detect the G211A mutants. PCR-RFLP is limited by its low throughput, high time-consuming for post-amplification manipulation, and generation of

false-positive results from partial restriction enzyme digestion or variants near the mutation of interest (Liebman et al., 1996; Harth et al., 2001). PCR with the Taqman MGB SNP assay has the advantages of simplicity, high throughput, and speed, but is considerably more expensive than DNA melting curve analysis with the FRET assay. Although the nucleotide substitution of glycine for arginine is easily genotyped by HRM, the efficacy of HRM has not been reported for detection of the G211A mutation. DNA melting curve analysis with the FRET assay, therefore, potentially offers two benefits. First, this method is performed without any post-PCR processing and, thus, eliminates the risk of carry over contamination from PCR products, low workforce, and additionally saves time (analysis is done within 2 h). Second, it enables the clinical molecular diagnosis of Gilbert's syndrome with high throughput analysis at a low cost. We argue, therefore, that this technique is optimal for the practical screening of the genetic polymorphisms and mutations within the *UGT1A1* gene.

Gilbert's syndrome sometimes coexists with other serious conditions and consequently can interfere with their diagnosis to cause unwarranted anxiety and fear in affected patients suffering of liver disease, especially in hyperendemic areas of viral hepatitis. Furthermore, patients with Gilbert's syndrome are at increased risk of developing drug toxicity from irinotecan therapy, which is used for the treatment of metastatic colorectal cancer (Schulz et al., 2009). Results obtained from DNA melting curve analysis could, therefore, offer a basis for accurate genetic counseling, improved diagnostic efficiency, and personalized medical therapy with anti-cancer agents, both in Caucasian and Asian populations.

In conclusion, DNA melting curve analysis represents a 3 "R" (rapid, robust, and reliable) alternative approach to direct sequencing while simultaneously and cost-effectively screening for the TATA-box polymorphism and G211A mutation of the *UGT1A1* gene.

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