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Rapid Allelic Discrimination by TaqMan PCR for the Detection of the Gilbert's Syndrome Marker UGT1A1*28

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Gilbert's syndrome causes mild, unconjugated hyperbilirubinemia and is present in approximately 10% of the Caucasian population. The basis of the disorder is a 70% reduction in bilirubin glucuronidation catalyzed by the UDP-glucuronosyltransferase 1A1 (UGT1A1), which, in Caucasians, is the result of a homozygous TA insertion into the promoter region of the UGT1A1 gene (UGT1A1*28). Homozygous carriers of UGT1A1*28 as well as those with additional UGT1A variants can suffer from severe irinotecan toxicity or jaundice during treatment with the protease inhibitor atazanavir. UGT1A1*28 genotyping identifies patients at risk for drug toxicity and can increase drug safety by dose individualization. Rapid and facile UGT1A1*28 genotyping is therefore of great clinical importance. Two hundred ninety-one patients with suspected Gilbert's syndrome were genotyped using the TaqMan 5' nuclease assay with minor groove binder-non fluorescent quench probes; results were confirmed by direct sequencing. Ninety-six patients (33%) were homozygous for UGT1A1*28, which was verified by direct sequencing of a different PCR product showing 100% concordance with the TaqMan PCR results. We describe a novel UGT1A1*28 genotyping method that employs allelic discrimination by TaqMan PCR. This assay provides a rapid, high-throughput, and cost-effective method for Gilbert's syndrome genotyping, which is of value for pretreatment screening of potential irinotecan toxicity. The method utilizes a technological platform that is widely used in clinical practice and could therefore be easily adapted for routine clinical applications. (J Mol Diagn 2008, 10:549–552; DOI: 10.2353/jmoldx.2008.080036)

Gilbert's syndrome is a common cause of mild jaundice that results from unconjugated hyperbilirubinemia due to reduced glucuronidation of bilirubin by UDP-glucuronosyltransferase 1A1 (UGT1A1).^{1,2} From a hepatological point of view Gilbert's syndrome is considered benign because it does not lead to chronic liver dysfunction or fibrosis. However, recent reports have linked it to drug toxicity and cancer predisposition. Patients treated with the protease inhibitor atazanavir, or the anti-tumor agent irinotecan are at an increased risk of developing jaundice or severe drug toxicity.^{3,4} Furthermore, an association of Gilbert's syndrome with breast cancer has been reported.⁵ Beyond identifying the nature of mild unconjugated hyperbilirubinemia UGT1A1*28 genotyping is a tool to predict drug side effects allowing for individualized therapeutic regimens with less toxicity.

The genetic basis of Gilbert's syndrome in Caucasians is a homozygous TA insertion into the TATA box promoter region of the UGT1A1 gene (UGT1A1*28) on chromosome 2. This variant exhibits 7 TA repeats (TA)₇ instead of the wild-type 6 TA repeats (TA)₆ leading to a 70% to 80% reduction of bilirubin glucuronidation in homozygous UGT1A1*28 carriers. This feature carries a high relevance because of its frequency amounting to approximately 0.4 in the Caucasian population, which would lead to 16% homozygous carriers.^{1,6}

Additional single nucleotide polymorphisms within the human UGT1A gene locus may also contribute to the phenotype of Gilbert's syndrome. A G71R exon variant with a frequency of approximately 0.23 (UGT1A1*6) has been described in Asians.⁷ An additional TA insertion in the promoter region leads to eight TA repeats resulting in a further reduction of glucuronidation activity has been

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Table 1. Taqman Probes and Primers

	Sequence	Concentration
Primer forward	5'-AACATTAACCTGGTGTATCGATTGGT-3'	600 nmol/L
Primer reverse	5'-AGCAGGCCCCAGGACAAAGT-3'	300 nmol/L
Probe (TA) ₆	6-FAM-5'-TTGCCATATATATATATATAAGTAGGA-3'-MGB	80 nmol/L
Probe (TA) ₇	VIC-5'-TGCCATATATATATATATATAAGTAGGA-3'-MGB	250 nmol/L

mainly detected in Africans (*UGT1A1**37, frequency up to 0.07 in Africans), and a promoter variant lacking one TA repeat [(TA)₅, *UGT1A1**36, frequency up to 0.08 in Africans] has been identified, which interestingly leads to an increased transcriptional activity.⁸ However, these variants are extremely rare in Caucasians.

Thus methodology capable of rapid and reliable detection of *UGT1A1**28 is of value. Several methodological approaches have been recently published, which include pyrosequencing,⁹ commercially available Invader™ assays approved by the Food and Drug Administration,¹⁰ the smart amplification process,¹¹ capillary electrophoresis and DNA fragment analysis,¹² or fully denaturing high-performance liquid chromatography.^{13,14} However, direct sequencing of the *UGT1A1* promoter region—although time consuming—remains the gold standard method for genotyping the *UGT1A1**28 variant.

Case Reports

Three patients receiving anticancer treatment with irinotecan were presented for a consultation by our outpatient oncology unit with the question of the presence genetic markers of Gilbert's syndrome. A 62- and a 72-year-old man with colorectal cancer and pulmonary as well as hepatic metastases had developed severe leukopenia, anemia, and diarrhea. A 72-year-old woman with gastric carcinoma and pulmonary as well as hepatic metastases had developed anemia and diarrhea under treatment requiring dose reductions and hospital admission for further treatment. Genotyping by direct resequencing of the *UGT1A1* gene promoter showed a homozygous TA insertion (*UGT1A1**28) in all three individuals, confirming the Gilbert's syndrome polymorphism. Based on these examples the issue of pretreatment *UGT1A1**28 screening of candidates for irinotecan therapy was raised in our tumor conference, and we were asked to suggest a rapid and facile genotyping test.

Materials and Methods

The TaqMan 5'-nuclease assay is an established method widely used for real-time PCR quantification. The design of genotype-specific probes allows a rapid allelic discrimination genotyping analysis. For other common single nucleotide polymorphisms of the *UGT1A* gene locus TaqMan assays either have been published or are commercially available. However, a method using TaqMan PCR for allelic discrimination of the clinically most important variant—*UGT1A1**28—has not been described so far.

Genomic DNA was isolated from whole blood samples of patients referred for suspected Gilbert's syndrome by the NucleoSpin Blood XL Kit according to the recommendations of the manufacturer (Machery and Nagel, Dueren, Germany). Approximately 40 ng were used as a template in TaqMan 5' nuclease assay. Primers and probes specific for (TA)₆ and (TA)₇, respectively, were designed with the Primer Express software (Applied Biosystems, Darmstadt, Germany) and labeled at the 3' end with either 6-carboxyfluorescein or VIC as reporter dyes and at the 5' end with a non-fluorescent quencher and a minor groove binding molecule (Applied Biosystems), the respective concentrations were optimized for allelic discrimination and are reported in Table 1. The detection run consisted of a hot start at 95°C for 10 minutes and 55 cycles of 94°C for 15 seconds and 60°C for 1 minute. All assays were performed as 25-μl reactions using qPCR Mastermix Plus (Eurogentec, Seraing, Belgium) supplemented with 600 μmol/L magnesium chloride to a final concentration of 5.6 mmol/L of magnesium chloride on 96-well plates using an ABI 7000 instrument (Applied Biosystems).

Results were confirmed by direct DNA sequencing after amplification of an 184-bp product of the *UGT1A1* promoter region followed by cycle sequencing. Primer sequences were 5'-TCCCTGCTACCTTTGTGGAC-3' (forward and sequence PCR) and 5'-AGCAGGCCCCAGGACAAAGT-3' (reverse). Sequence PCR was performed using the Dye Terminator Cycle Sequencing Kit 1.1 (Applied Biosystems). The nucleotide sequences were determined on an ABI 310 automated sequencer (Applied Biosystems).

As the promoter region of the *UGT1A1* gene consists of six or seven TA repeats *UGT1A1**28 represents a challenge for probe design. Factors that complicate TaqMan probe binding and detection include insertional mutations that enable binding of the non-specific probe by causing hairpin structures, as well as multiple TA repeats leading to formation of probe homo- and heterodimers, which can inhibit binding to the target sequence. The following adjustments had to be made to the standard protocol to ensure an adequate efficiency.

TA repeats have a low binding affinity and therefore lead to a reduced binding temperature of short probes. Long TaqMan probes are less suitable for allelic discrimination because the mismatching oligonucleotide(s) have to contribute substantially to the binding affinity to allow sufficient discrimination between the differing genotypes. We therefore used minor groove binder-non fluorescent quench (Applied Biosystems) to gain a higher binding temperature with short probes. Due to different binding kinetics of the wild-type and variant probe, the respective

Figure 1. Typical example of the allelic discrimination of the *UGT1A1**28 variant by TaqMan PCR. NTC, no template control.

probe concentrations as well as the amount of primers had to be modified, and magnesium added to increase specificity. In addition, the PCR run had to be extended to 55 cycles to gain efficient differing fluorescent signals.

Results and Discussion

The resulting cluster plot shows strong fluorescent signals for each allele and a clear separation between the three clusters discriminating the variant, heterozygous, and *UGT1A1**1 allele status (Figure 1). The assay showed low interprobe variability with a maximum SD of 8.4% (Figure 2). Interassay variation for the same probes was higher indicating the need for additional genotyping of positive controls with each run since fluorescence levels alone cannot provide sufficient information on the respective genotype. Although this may not be necessary in most cancer center settings this method permits the analysis of up to 44 patients (duplicates including two no-template controls, and two positive controls for wild-type, heterozygous, and homozygous status) in a single run using a standard 96-well-plate.

Two hundred and ninety-one patients with isolated raised bilirubin levels and therefore suspected Gilbert's syndrome were analyzed, of whom 96 (33%) were homozy-

gous and 100 (34.4%) heterozygous for the *UGT1A1**28 variant, and 92 (31.6%) were homozygous *UGT1A1**1. The results showed 100% concordance with independent DNA sequencing.

Three patients of North African descent with the rare *UGT1A1**36 promoter polymorphism characterized by five TA repeats (one homozygous variant carrier, two heterozygous carriers) were included in our study. In these patients very low reporter dye signals were recorded, which were only slightly higher than those of the no-template controls (Figure 1), which suggests absent or very inefficient probe binding in the presence of the *UGT1A1**36 variant. The signal of the 6-Fam-labeled probe specific for the wild-type allele in patients with a heterozygous variant was not high enough to falsely identify them as wild type. Although other reasons for the failure of allelic discrimination including poor quality of DNA have to be excluded, the described method appears suitable for the detection of homozygous as well as heterozygous individuals with the rare *UGT1A1* (TA)₅ genotype. In these cases direct sequencing would have to ensue in case of low dye signals, which would be expected in very few patient samples. DNA from patients harboring the homozygous *UGT1A1**37 variant (8 TA repeats) was not available and therefore not included in our study. As most of the available rapid genotyping methods for *UGT1A1* variants are not suitable for detection of *UGT1A1**37, the rare *UGT1A1* (TA)₈ genotype remains limited to direct sequencing.

Conclusions

The clinical need for genotyping for the (TA)₇ variant of the *UGT1A1* promoter region is being increasingly recognized as a predictive factor of standard therapy with certain drugs such as atazanavir and irinotecan. The Food and Drug Administration has already recommended a reduced initial dose of the anti-tumor agent irinotecan for patients known to be homozygous for the *UGT1A1**28 allele.¹⁵ In a recent meta-analysis of nine studies with a total of 821 irinotecan-treated patients an association of toxicity with *UGT1A1**28 was confirmed, but it was also noted that in patients receiving lower doses of irinotecan (100 to 125 mg/m²) the risk of side effects was similar in *UGT1A1**1 and *UGT1A1**28 carriers.¹⁶ The use of TaqMan allelic discrimination for *UGT1A1**28 genotyping is of special interest as the methodology is in frequent use in clinical laboratories and could be adapted from those using the TaqMan for real-time PCR technology. Recent reports have shown that additional polymorphisms of the *UGT1A* gene locus are important and can also be detected by adapting the aforementioned methodology (described in³). Against this background the described methodology would provide a facile and robust method suitable for high volume cohorts. In our case rapid and facile pre-treatment genotyping in candidate patients for irinotecan therapy could be implemented using the above outlined methodology.

Figure 2. Analysis of 10 samples each of one wild-type, one heterozygous, and one homozygous probe shows low interprobe variability. Mean values for VIC and FAM fluorescence are shown for each probe with bars representing mean SD.

References

1. Bosma PJ, Chowdhury JR, Bakker C, Gantla S, de Boer A, Oostra BA, Lindhout D, Tytgat GNJ, Jansen PLM, Oude Elferink RPJ, Chowdhury NR: The genetic basis of the reduced expression of bilirubin UDP-glucuronosyltransferase 1 in Gilbert's syndrome. *N Engl J Med* 1995, 333:1171-1175
2. Strassburg CP, Manns MP: Jaundice, genes and promoters. *J Hepatol* 2000, 33:476-479
3. Lankisch TO, Moebius U, Wehmeier M, Behrens G, Manns MP, Schmidt RE, Strassburg CP: Gilbert's disease and atazanavir: from phenotype to UDP-glucuronosyltransferase haplotype. *Hepatology* 2006, 44:1324-1332
4. Ando Y, Saka H, Ando M, Sawa T, Muro K, Ueoka H, Yokoyama A, Saitoh S, Shimokata K, Hasegawa Y: Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. *Cancer Res* 2000, 60:6921-6926
5. Guillemette C, Millikan RC, Newman B, Housman DE: Genetic polymorphisms in uridine diphospho-glucuronosyltransferase 1A1 and association with breast cancer among African Americans. *Cancer Res* 2000, 60:950-956
6. Bosma PJ: Inherited disorders of bilirubin metabolism. *J Hepatol* 2003, 38:107-117
7. Aono S, Adachi Y, Uyama E, Yamada Y, Keino H, Nanno T, Koiwai O, Sato H: Analysis of genes for bilirubin UDP-glucuronosyltransferase in Gilbert's syndrome. *Lancet* 1995, 345:958-959
8. Beutler E, Gelbart T, Demina A: Racial variability in the UDP-glucuronosyltransferase 1 (UGT1A1) promoter: a balanced polymorphism for regulation of bilirubin metabolism?. *Proc Natl Acad Sci USA* 1998, 95:8170-8174
9. Skarke C, Grosch S, Geisslinger G, Lotsch J: Single-step identification of all length polymorphisms in the UGT1A1 gene promoter. *Int J Clin Pharmacol Ther* 2004, 42:133-138
10. Hasegawa Y, Sarashina T, Ando M, Kitagawa C, Mori A, Yoneyama M, Ando Y, Shimokata K: Rapid detection of UGT1A1 gene polymorphisms by newly developed Invader assay. *Clin Chem* 2004, 50:1479-1480
11. Watanabe J, Mitani Y, Kawai Y, Kikuchi T, Kogo Y, Oguchi-Katayama A, Kanamori H, Usui K, Itoh M, Cizdziel PE, Lezhava A, Tatsumi K, Ichikawa Y, Togo S, Shimada H, Hayashizaki Y: Use of a competitive probe in assay design for genotyping of the UGT1A1*28 microsatellite polymorphism by the smart amplification process. *Biotechniques* 2007, 43:479-484
12. Huang CK, Dulau A, Su-Rick CJ, Pan Q: Validation of rapid polymerase chain reaction-based detection of all length polymorphisms in the UGT 1A1 gene promoter. *Diagn Mol Pathol* 2007, 16:50-53
13. Pirulli D, Giordano M, Puzzer D, Crovella S, Rigato I, Tiribelli C, Momigliano-Richiardi P, Amoroso A: Rapid method for detection of extra (TA) in the promoter of the bilirubin-UDP-glucuronosyl transferase 1 gene associated with Gilbert syndrome. *Clin Chem* 2000, 46:129-131
14. Harraway JR, George PM: use of fully denaturing HPLC for UGT1A1 genotyping in Gilbert syndrome. *Clin Chem* 2005, 51:2183-2185
15. O'Dwyer PJ, Catalano RB: Uridine diphosphate glucuronosyltransferase (UGT) 1A1 and irinotecan: practical pharmacogenomics arrives in cancer therapy. *J Clin Oncol* 2006, 24:4534-4538
16. Hoskins JM, Goldberg RM, Qu P, Ibrahim JG, McLeod HL: UGT1A1*28 genotype and irinotecan-induced neutropenia: dose matters. *J Natl Cancer Inst* 2007, 99:1290-1295