A clinical PCR fragment analysis assay for TA repeat sizing in the UGT1A1 promoter region

Ahmad N. Abou Tayoun, Francine B. de Abreu, Joel A. Lefferts, Gregory J. Tsongalis *

Department of Pathology, Geisel School of Medicine at Dartmouth, Hanover, NH, United States
Dartmouth Hitchcock Medical Center and Norris Cotton Cancer Center, Lebanon, NH, United States

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A B S T R A C T
Background: A genetic TA repeat length polymorphism in the UGT1A1 promoter has been shown to affect UDP-glucuronosyltransferase (UGT1A1) expression levels with significant clinical implications. The presence of 7 TA repeats has been associated with lowered UGT1A1 expression and the mild hyperbilirubinemia manifested in Gilbert’s syndrome. Furthermore, cancer patients carrying this variant exhibit irinotecan-related toxicity and require lower doses of this chemotherapeutic agent compared to patients carrying the 6 TA repeat allele. This polymorphism is very common and, therefore, necessitates the development of reliable means of detecting it in the clinical laboratory to deliver better personalized therapy regimens.

Methods: We used 45 whole blood samples from patients previously tested with the FDA-approved Invader UGT1A1 assay (Hologic, Madison, WI) to assess extraction method, analytical sensitivity, accuracy and precision of this assay. In addition, cell line controls were used to test for the common and rare alleles of this polymorphism. The assay was based on PCR amplification and capillary electrophoresis for accurate sizing of the TA repeat copy number.

Results: All samples tested and controls gave the expected results.

Conclusions: We have developed and validated a simple and sensitive PCR fragment analysis assay for accurately determining TA repeat length in the UGT1A1 promoter. At our medical center, this testing is used primarily for guiding irinotecan dosing decisions for our cancer patients.

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1. Introduction

A strikingly successful example in pharmacogenetics and personalized medicine fields is the use of genetic polymorphisms in the UGT1A1 gene to aid diagnosis and guide individualized therapies. Uridine diphosphate (UDP)-glucuronosyltransferase (UGT1A1) plays an important role in the metabolism of drugs and endogenous substances through glucuronidation. Genetic variations in the promoter of the UGT1A1 gene – mainly the TA repeat polymorphism – have been reported to affect its expression leading to significant alterations in its immediate targets and serious clinical manifestations [1–4]. For example, there is a direct link between the mild hyperbilirubinaemia seen in Gilbert’s syndrome and homozygosity for a 2 bp (TA) insertion in the UGT1A1 promoter [1]. This insertion has been shown to reduce UGT1A1 expression and leads to the deficiency in bilirubin glucuronidation manifested in Gilbert’s syndrome [1]. The presence of this insertion in the UGT1A1 promoter increases TA repeats to seven (designated as UGT1A1*28) and is currently being used to confirm a diagnosis of Gilbert’s syndrome.

There has also been strong clinical evidence associating a decrease in UGT1A1 activity and irinotecan-related toxicity [3]. Irinotecan is currently used in first line chemotherapy [5,6] with UGT1A1 playing an important role in metabolizing its active metabolite, SN-38. A decrease in SN-38 glucuronidation accompanying UGT1A1 reduction increases the severity of irinotecan-induced diarrhoea [2,7]. In fact, to support oncologists’ endeavors in providing safe and individualized irinotecan doses for cancer patients, the US Food and Drug Administration (FDA) cleared a UGT1A1 assay for detecting TA polymorphism based on the Invader molecular technology (Hologic, Madison, WI) [8]. Unlike cancer patients homozygous for six TA repeats in the UGT1A1 promoter (designated as UGT1A1*1), patients homozygous or heterozygous for the UGT1A1*28 allele have reduced UGT1A1 expression and are more likely to receive lowered irinotecan doses to avoid potential toxic side effects. The UGT1A1*28 allele frequency can be as high as 39% and 43% in the Caucasian and African populations, respectively [9]. This together with the associated clinical implications demands the development of simple but reliable means of detecting this allele in the clinical laboratory. There has also been suggestion that the FDA approved assay will no longer be commercially available in the near future. Here, we present clinical validation data on a PCR fragment analysis assay developed for determining TA polymorphism status in the UGT1A1 promoter.
2. Materials and methods

2.1. DNA samples

A total of 50 samples were used in this study. Genomic DNA from 45 patient blood samples was extracted using the Qiagen EZ1 robotic system or the QIAamp DNA kit (Qiagen, Valencia, CA). All 45 samples were genotyped for the UGT1A1 TA repeat number using the Invader® UGT1A1 Molecular Assay (Hologic, Madison, WI). In addition, DNA from 5 cell lines of known genotypes was obtained from the Coriell Institute (NA17039, NA17119, NA17120, NA17139, NA17438) and used in assessing the performance of our new assay. DNA was quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA).

2.2. PCR conditions

Primers flanking the TA repeat polymorphism site in the UGT1A1 promoter [10] were ordered from Integrated DNA Technologies (Coralville, Iowa) and used for PCR amplification. The reverse primer was 5′-FAM labeled for subsequent amplicon detection during capillary electrophoresis (see below). The primer sequences were compared in silico to human genomic DNA sequences within the NCBI databases. No significant similarities between the targeted sequences in the UGT1A1 gene and the rest of the human genome that could theoretically result in non-specific amplification and detection were found.

We prepared 25 μL PCR reactions consisting of 1X GeneAmp® PCR Gold buffer (Life Technologies), 3.5 mM MgCl₂, 0.2 mM dNTPs, 0.25 μM UGT1A1 forward primer (5′-AAGTGAATCCCTGCTACCTT-3′), 0.25 μM 5′-labeled UGT1A1 reverse primer (5′-FAM/CCACTGGGATCAAGACTCTTACCT-3′), 1 unit of the AmpliTaq Gold® DNA polymerase (Life Technologies), and genomic DNA (0.1-180 ng/μL). PCR amplification was performed on the Bio-Rad S1000 thermal cycler (Bio-Rad, Hercules, CA) and included 5 minute hot start (95 °C) followed by 40 cycles of 30 second denaturation (95 °C) and 45 second annealing/extension at 55 °C. A final 20 minute extension at 72 °C was also included. PCR amplification was confirmed using gel electrophoresis.

2.3. Capillary electrophoresis

The FAM-labeled PCR product was diluted 1/10 and treated with Hi-Di Formamide and GeneScan™-500 ROX™ size standard (Applied Biosystems, Carlsbad, CA) for capillary electrophoresis and fragment analysis using the 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Peaks with their corresponding sizes were used to deduce TA repeat size and allele status (Fig. 1).

3. Results

To determine TA repeat size in the UGT1A1 promoter, we first amplified the region encompassing this polymorphism site using PCR. A fluorescent dye was incorporated into the amplified product by adding 6-carboxyfluorescein (6-FAM™) to the 5′ end of the reverse primer. This PCR product was subsequently subjected to capillary electrophoresis and fragment analysis to accurately determine the TA repeat number as shown in Fig. 1.

3.1. DNA extraction

To examine the compatibility of our assay with different extraction protocols, we extracted genomic DNA from anticoagulated whole blood (EDTA or ACD) patient samples using either the Qiagen EZ1 robotic system (21 samples) or the QIAamp column DNA kit (45 samples) that is routinely used in the clinical laboratory for DNA-based molecular diagnostics. Our assay showed robust performance using DNA extracted with either system and was able to correctly identify UGT1A1 TA repeat sizes in all samples tested in this extraction study.

3.2. Analytical sensitivity

We assessed our assay’s limit of detection (LOD) by varying the amount of input genomic DNA used upfront in the PCR reaction. Interestingly, our assay was able to give correct genotype calls using as low as 0.5 ng input DNA (Fig. 2). However, since the electropherograms started losing signal intensity at 1 ng or less (Fig. 2) we established the LOD at 5 ng input DNA. We confirmed this by correctly identifying TA repeat sizes in 21/21 samples (100%) using 5 ng of genomic DNA.

3.3. Accuracy

The accuracy of the UGT1A1 fragment analysis assay was validated using a total of 50 samples (Table 1) divided into three groups.

The first group consisted of 24 DNA samples that were previously extracted from EDTA peripheral blood using the QIAamp DNA kit. All 24 DNA samples, previously genotyped using the Invader® UGT1A1 Assay, were blindly analyzed using our UGT1A1 fragment analysis assay. The results revealed 100% concordance in the genotypes obtained using the two different assays. The 24 samples included the following genotypes: 6/6 (n = 10), 6/7 (n = 12) and 7/7 (n = 2) (see also Table 1).

The second group consisted of 12 ACD and 9 EDTA peripheral blood samples. DNA from all 21 blood samples were extracted using the QIAamp DNA kit and then subsequently genotyped using the Invader® UGT1A1 Assay. DNA from the same 21 blood samples were then extracted using the EZ1 whole blood extraction system and were subjected to the UGT1A1 fragment analysis assay. Comparison of the genotypes obtained using both assays showed that 20/21 samples were similarly called. One sample was genotyped by the Invader assay as “Other” indicating that a 6/6, 6/7, or 7/7 genotype was not detected in the sample which was found to be “5/7” using our UGT1A1 fragment analysis assay. Hence, the genotypes included in this set were: 6/6 (n = 9), 6/7 (n = 9), 7/7 (n = 2), and 5/7 (n = 1) (see also Table 1).

Finally, the third group consisted of 5 DNA samples with rare genotypes obtained from the Coriell Institute for Medical Research. Samples were blindly genotyped using our fragment analysis assay. All genotypes were correctly identified: 5/6 (NA17039), 6/8 (NA17119), 5/7 (NA17120), 5/8 (NA17139), and 7/8 (NA17438). In summary, our assay correctly identified TA repeat sizes in all 49/49 tested samples for a 100% accuracy (Table 1).

3.4. Precision

Eight samples with common and rare genotypes (6/6, 6/7, 7/7, 5/7, 5/8) were tested in duplicate on multiple days to examine the precision of our assay. Reproducibility or the lack of inter-assay/within-assay variability was demonstrated when identical calls (100% precision) were made for the 8 duplicate samples at five independent runs generated by different operators.

4. Discussion

The data presented here show that our newly developed PCR fragment analysis assay provides a highly sensitive means of accurately determining UGT1A1 promoter TA repeat size. This new assay is based on PCR and capillary electrophoresis that are routinely used in standard molecular diagnostic laboratories.

Unlike the Invader® UGT1A1 Molecular Assay which detects only 6/6, 6/7 and 7/7 genotypes, our assay has the distinct ability to detect potentially any TA repeat number in the UGT1A1 promoter present as homozygous or in combination with other TA repeat size alleles. Here,
we were able to accurately and precisely detect 5, 6, 7 and 8 TA repeats in patient and cell line DNA samples that were homozygotes or compound heterozygotes (Table 1). Although the clinical significance of the 5 and 8 TA repeat alleles is not well-established, there is clear evidence that TA repeat size inversely affects UGT1A1 expression [9]. This implies that patients carrying the 8 TA repeat allele might have
the lowest UGT1A1 expression levels and, therefore, might require special care if they show the relevant clinical manifestations. This is especially the case for patients of African descent since the 5 and 8 TA allele frequencies are 3.5% and 6.9% in that population, respectively [9]. Perhaps the main limitation of our assay is the inability to detect other UGT1A1 variants outside the TA polymorphism targeted site that could potentially disrupt UGT1A1 expression levels or enzyme activity. However, unlike the rare Crigler–Najjar syndrome where severe hyperbilirubinemia is caused by mutation in UGT1A1 coding sequence, the clinical diagnosis of Gilbert’s syndrome has not been associated with variants aside from the more commonly inherited promoter TA polymorphism [4]. Therefore, expanding UGT1A1 variant detection might not be necessary or cost-effective for the diagnosis of Gilbert’s syndrome. In our clinical setting, UGT1A1 genotyping is used most often for guiding irinotecan therapy decisions. In making the decision to include rare UGT1A1 variants in testing for irinotecan dose specification, the clinical significance and the frequency of such variants in the general population have to be carefully investigated in order to maintain cost-effective diagnostic tools that can deliver clinically actionable information.

Conflict of interest statement

The authors have no conflict of interest with respect to this work.

Acknowledgments

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References


Table 1

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