Short Communication

Development of a rapid clinical TPMT genotyping assay

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ABSTRACT

Objectives: Thiopurine compounds are commonly used in the treatment of childhood acute lymphoblastic leukemia, and as immunosuppressants following organ transplantation or for treatment of various autoimmune disorders. Thiopurine S-methyltransferase (TPMT) is required for detoxification, through S-methylation, of 6-thioguanine nucleotides (TGNs), a byproduct of thiopurine metabolism. Single nucleotide polymorphisms (SNPs) in the TPMT gene have been shown to affect its function, with some variants associated with serious clinical manifestations including severe to fatal myelosuppression and organ transplant rejection following treatment with standard thiopurine doses. In this study, we describe a TaqMan real time PCR allelic discrimination assay requiring minimal DNA input for TPMT genotyping.

Design and methods: We designed controls for the homozygous wild type and allelic variants of TPMT*2, *3B, and *3C. Genomic DNA was extracted from an additional 412 human blood samples. The samples were tested for the TPMT*2, *3B, *3C, and *3A polymorphisms by TaqMan genotyping assays using the AB 7500 FAST Real-Time PCR instrument. Allelic discrimination plots were used to identify each mutation.

Results: The TaqMan assay correctly genotyped all custom control DNA samples. Of the 412 tested samples, our assay identified 375 samples as wild-type *1/*1 (91.02%), 3 as *1/*2 (0.73%), 1 as *1/*3B (0.24%), 3 as *1/*3C (0.73%), 27 presumed to be *1/*3A (6.55%), and 3 as *3B/*3A (0.73%).

Conclusions: The clinical implications of TPMT genotyping, along with the simplicity and specificity of the TaqMan genotyping assays make this test highly suitable for use in a clinical laboratory.

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Introduction

Thiopurine drugs are commonly used as immunosuppressants following organ transplantation and in the treatment of patients with childhood acute lymphoblastic leukemia, and as immunosuppressants following organ transplantation or for treatment of various autoimmune disorders (e.g., Crohn’s disease, rheumatoid arthritis) [1,2]. Thiopurine drugs undergo several in vivo enzymatic modifications resulting in the formation of 6-thioguanine nucleotides (TGNs) [2]. TGNs are antimetabolites that incorporate into DNA during cell division, subsequently disrupting nucleic acid synthesis and triggering cell apoptosis [2,3]. The toxic effects of TGNs are normally obstructed by detoxification pathways catalyzed by xanthine oxidase, Thiopurine S-methyltransferase (TPMT), and aldehyde oxidase [5]. TPMT’s essential role in the S-methylation of thiopurines yields 6-methyl-MP, 6-methyl-thioguanine, and other less cytotoxic substances [3,4,6]. This methylation greatly reduces the toxic side effects of thiopurine drugs, making them better suited for treatment.

The TPMT gene is localized to chromosome 6p22.3 and is approximately 34 kb in length, containing 9 introns and 10 exons [7]. It has an open reading frame (ORF) of 735 bp that encodes for a 245-amino acid peptide [2]. The wild type TPMT allele, expressing the enzyme with normal activity, has been designated TPMT*1. To date, there have been 30 variant TPMT alleles described [8]. Of those 30 alleles, 4 account for more than 90% of the TPMT forms with low activity; TPMT*2, TPMT*3B, TPMT*3C, and TPMT*3A [9,13]. The allelic frequency of each of these variants, within Caucasian, African, and Asian populations is 0.2–0.4% for TPMT*2 (rs1800462), 0.0–2.6% for TPMT*3B (rs1800460), 1.2–6.2% for TPMT*3C (rs1142345), and 0–5.7% for TPMT*3A [8,9]. The TPMT*2 (NM_000367.2:c.238G>C; NP_000358.1: p.Ala80Pro), TPMT*3B (NM_000367.2:c.460G>A; NP_000358.1: p.Ala154Thr), and TPMT*3C (NM_000367.2:c.719A>G; NP_000358.1: p.Tyr240Cys) alleles are single nucleotide polymorphisms (SNPs) in exons 5, 7, and 10, respectively (Fig. 1A) [3]. The TPMT*3A allele includes the variants found in both the TPMT*3B and TPMT*3C alleles (460G>A and 719A>G) [3].
Patients heterozygous for any one of the above \( TPMT \) variants have intermediate \( TPMT \) activity. The carrier frequency of allelic variants in a Caucasian population can reach 11%. Homozygous or compound heterozygous patients have extremely low to no \( TPMT \) activity and occur at a frequency of up to 0.6% in the general population [3,11]. Patients with reduced \( TPMT \) activity can suffer from unanticipated severe to fatal myelosuppression and organ transplant rejection when treated with standard doses of thiopurine drugs, due to the patient’s inability to detoxify TGNs [6,12]. Recent studies suggest that providers should reduce the standard thiopurine dosage by 50–67% for patients heterozygous for any of the above \( TPMT \) variants [13]. Guidance on the use of thiopurines on homozygous or compound heterozygous patients is unclear, with some studies suggesting that thiopurine use is contraindicated and yet homozygous patients have shown successful responses to low thiopurine amounts that are less than 20% the standard dosage [13].

Previously described \( TPMT \) genotyping methods include polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis, real-time PCR with melt curve analysis, single strand conformational polymorphism (SSCP) analysis, amplification refractory mutation system (ARMS), hybridization probe assays, and pyrosequencing [3,13]. In this study, we describe a new allelic discrimination assay including the design of two custom primers and probes that requires minimal DNA input, and generates fast, easily interpretable TaqMan real-time PCR results for \( TPMT \) genotype.

**Materials and methods**

**Controls**

We designed synthetic, double-stranded DNA controls for the wild type and allelic variants of \( TPMT*2 \), \( *3B \), and \( *3C \) using Integrated DNA Technologies, Inc. (IDT® Coralville, IA) (Table 1). Equal concentrations of each wild type control were combined to make a \( TPMT \) wild type control mixture. Similarly, equal concentrations of each allelic variant control were combined to create one \( TPMT \) allelic variant control mixture. The \( TPMT \) heterozygous control was made by combining equal concentrations of wild type and variant allele controls for each variant within the same mixture.

**Samples**

In this study, we used 412 de-identified, unselected, remnant patient DNA samples obtained from EDTA whole blood from a predominantly Caucasian population at Dartmouth Hitchcock Medical Center (Lebanon, NH). Genomic DNA was isolated using the EZ 1 Robotic System (Qiagen, Valencia, CA).

**TaqMan Allelic Discrimination Assay**

In order to have knowledge of the primer and probe sequences we designed TaqMan Allelic Discrimination Assays for \( TPMT*2 \) and \( TPMT*3C \) (Table 2), and used one previously designed for \( TPMT*3B \) (Life Technologies TaqMan SNP Genotyping Assay ID number C__30834416__20). The TaqMan Allelic Discrimination Assays were performed using the 412 DNA samples in MicroAmp Fast Optical 96-well Reaction Plates sealed with MicroAmp Optical Adhesive Film. Each 10 \( \mu \)L reaction mixture contained 5 \( \mu \)L of 2X TaqMan GTXpress Master Mix, 0.25–0.5 \( \mu \)L of the appropriate 40–20X Primer Probe Mix, respectively, and 2 ng of DNA. Amplification and allelic discrimination were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The PCR amplification profile was as follows: pre-PCR read at 60 °C for 1 min, followed by initial denaturation at 95 °C for 20 s, then 40 cycles of denaturation at 92 °C for 3 s and annealing/extension at 60 °C for 30 s, and post-PCR read at 60 °C for 1 min. Data were collected during the pre-PCR read and post-PCR read, and during thermocycling at the annealing/extension step only. The pre and post-PCR plate read was used for allelic discrimination using 7500 Software v2.0.5.

**Commercial TaqMan Allelic Discrimination Assay comparison**

To verify the accuracy of the custom primers and probes a subset of 10 patient samples was additionally assayed via commercially available TaqMan primers and probes from Life Technologies. The reaction and PCR settings were performed as above.

**Results**

To determine the genotype of each sample, we used TaqMan assays to amplify three exonic regions encompassing the above-mentioned SNPs. Each assay used 2 ng of DNA and included specific primers and differentially labeled probes for the wild type and variant alleles (Fig. 1B).

As an initial assay validation step we correctly identified all custom designed control DNA samples using our allelic discrimination assays.

For the \( TPMT*2 \) allelic discrimination assay, 409 samples were identified as homozygous wild-type (99.27%), 3 as heterozygous (0.73%), and none as homozygous \( TPMT*2 \). For the \( TPMT*3B \) allelic discrimination assay, 381 samples were identified as homozygous wild-type (92.48%), 28 as heterozygous (6.80%), and 3 as homozygous \( TPMT*3B \) (0.73%). For the \( TPMT*3C \) allelic discrimination assay, 379
samples were identified as homozygous wild-type (91.99%), 33 as heterozygous (8.01%), and none as homozygous TPMT*3C.

The three samples heterozygous for TPMT*2 were identified as homozygous wild-type for the TPMT*3B and TPMT*3C variants. Of the 28 samples identified as heterozygous for TPMT*3B, 27 were found to also be heterozygous for TPMT*3C, and thus presumed to be *1/*3A. The three samples homozygous for the TPMT*3B variant were found to be heterozygous for TPMT*3C, consistent with a *3B/*3A genotype.

In all, our assay identified 375 samples as wild-type *1/*1 (91.02%) with respect to *2, *3A, *3B, and *3C, 3 as *1/*2 (0.73%), 1 as *1/*3B (0.24%), 3 as *1/*3C (0.73%), 27 as presumed to be *1/*3A (6.55%), and 3 as *3B/*3A (0.73%).

To verify the accuracy of the custom designed primers and probes we compared the genotype of a ten patient subset to the genotypes determined via an assay using commercially available TaqMan primers and probes. The tenth sample however produced a discordant genotype and this discordance was not elucidated further as the treatment protocol for either of these two genotype possibilities would be identical.

Discussion

In this study, we report on the validation of our TPMT genotyping method for the detection of the most common allelic variants affecting TPMT activity. Our assays are PCR-based and rely on differentially labeled probes for the simultaneous detection of both the wild type and variant alleles. Such assays are simple to develop and can be routinely performed on real-time PCR platforms that are available in most molecular diagnostic laboratories.

The results presented here showed 100% concordance with what was expected for the control DNA samples. In addition, the relative frequency of identified variants correlates closely with what is expected for a Caucasian population [10].

Although TaqMan’s high specificity and minimal DNA requirement come at a slightly greater financial cost due to the fluorescently labeled probes, it is relatively easier to design compared to intercalating-dye based real-time PCR. In the latter, primers have to be carefully designed not only to detect the variant allele of interest but also to be able to resolve the wild type and variant amplified products through high-resolution melt curve analysis.

The TaqMan assay also offers a rapid turnaround time (TAT) of 40 min, whereas most other TPMT genotyping methods require several hours.
hours [3]. A recent method has been described using real-time PCR with the Light Cycler® instrument, which can yield results in 70 min [3,14]. This is the most rapid TAT currently available for TPMT genotyping.

Another advantage of the TaqMan assay is its ease of iteration; probes can be changed 1 nucleotide at a time without changing the amplification conditions required for multiplexing with the TaqMan primer probe mixes. Probes could essentially be labeled with different fluorescent dyes and be placed in the same reaction setup to test for up to two TPMT mutations (4 probes per well) at a time. Further testing would have to be performed to show this but it is a very intriguing concept that could simplify this test even more and minimize cost.

One limitation of this assay is the inability to distinguish TPMT*3B/TPMT*3C compound heterozygotes from TPMT*3A heterozygotes. As indicated in our study, a patient who was identified as heterozygous for both TPMT*3B and *3C was deemed as a *3A variant. The variants associated with *3B and *3C could exist on the same allele, resulting in a heterozygous *1/*3A patient, or the two variants could be present on separate alleles, resulting in a compound heterozygous patient (*3B/*3C). With both variants located on the same allele the heterozygous *3A patient would only have one defective allele, whereas the compound heterozygous patient would have two defective alleles (*3B/*3C). A compound heterozygous patient would be given much lower thiopurine dosage than a patient heterozygous for these variants. In the cases where the location of each of these mutations cannot be determined through this assay alone, parental testing could be used to resolve the ambiguity. It is, however, relatively safe to assume *1/*3A since the frequency of a *3B/*3C genotype would be considerably lower than a *3A/TPMT*3A homozygote. TPMT enzymatic testing could be used to determine whether the patient is heterozygous for the *3A allele or compound heterozygous for the *3B and *3C alleles. TPMT enzymatic testing is available at national reference laboratories.

Alternatively, if parental testing is not feasible the measurement of TPMT enzyme activity could be used to determine whether the patient is heterozygous for the *3A allele or compound heterozygous for the *3B and *3C alleles. TPMT enzymatic testing is available at national reference laboratories.

The implications of TPMT genotyping, along with the simplicity and specificity of the TaqMan genotyping assay make this test highly suitable for use in a clinical laboratory.

References