Abstract. The aim of the study is to present a novel approach for preparing triple-compound heterozygous reference material (TCH-RM) for thiopurine S-methyltransferase (TPMT) genotyping by using the gene synthesis technology. The polynucleotide chain we prepared consisted of three wild-type and three mutant segments corresponding to the TPMT 238G>C, 460G>A, and 719A>G polymorphic sites. TCH-RM characteristics were assessed via four methods: reverse hybridization, real-time PCR with hydrolysis probes, real-time PCR followed by subsequent melting temperature analysis, and DNA sequencing. Consequently, we investigated the TPMT genotype of 371 patients suffering from autoimmune diseases requiring immunosuppressive therapy with thiopurine drugs, mostly inflammatory bowel disease. All methods confirmed the triple heterozygous character and commutability of TCH-RM. In evaluating its stability we obtained very comparable data before and after six months of storage at -80 °C. The determined genotypes were as follows: 352 wild-type subjects (94.8 %), 17 TPMT*3A heterozygotes (460G>A and 719A>G, 4.6 %), one patient heterozygous for the TPMT*2 allele (238G>C, 0.3 %), and one TPMT*3C heterozygote (719A>G, 0.3 %). The frequencies of TPMT*1, *3A, *3C, and *2 in the patients were 97.5 %, 2.3 %, 0.1 %, and 0.1 %, respectively. Assembling segments of synthetic DNA into long polynucleotide chains is a universal way of obtaining compound heterozygous material for performing any simultaneous analysis of polymorphic sites in the human genome. The batches are manufactured with a perfect concentration match of wild-type and mutant fragments, and can be made in large quantities for most diagnostic techniques.

Introduction

Thiopurine S-methyltransferase (EC 2.1.1.67, TPMT) is a cytosolic enzyme catalysing the S-methylation of thiopurine drugs (azathioprine, 6-mercaptopurine, 6-thioguanine). It is encoded by a 27 kb gene located on chromosome 6p22.3. Depending on the ethnicity, either low or intermediate TPMT activity was found in 5–11 % of the population (Gearry and Barclay, 2005), indicating hereditary polymorphism in the TPMT gene in humans. The wild-type TPMT allele was designated TPMT*1. The most frequent allelic variants associated with the altered enzyme activity are: TPMT*2 (c.238G>C, rs1800462), TPMT*3B (c.460G>A, rs1800460), TPMT*3C (c.719A>G, rs1142345), and TPMT*3A (c.460G>A and c.719A>G).

All the minor variants in the TPMT gene are considered to slow down the metabolism of thiopurine drugs in the body. TPMT enzymes encoded by these alleles have a significantly shorter half-life than the wild-type (Krynetski and Evans, 1999). The lower cellular levels observed are caused by enhanced proteasomal degradation of misfolded mutant TPMT (Tai et al., 1997).

Genetic testing applied prior to the first administration of thiopurines allows identification of up to 98 % of slow drug metabolizers (Yates et al., 1997). Moreover, it prevents drugs overdose and reduces the risk of developing severe myelosuppression, hepatic toxicity, pancreatitis, mucositis, or dermatological problems. Numerous methods have been previously used for TPMT genotyping, including restriction analysis (Yates et al., 1997), multiplex amplification refractory mutation sys-
tems (Roberts et al., 2004), single-strand conformational polymorphism (Spire-Vayron de la Moureyre et al., 1998a), real-time PCR (Schutz et al., 2000), reverse hybridization strips (Fakhoury et al., 2007), pyrosequencing (Haglund et al., 2004), and microarray technology (Soltysova et al., 2011).

The analytical accuracy and reliability of TPMT genotyping are commonly ensured by using reference control materials (RM) based on genomic DNA (Spire-Vayron de la Moureyre et al., 1998b), recombinant DNA molecules obtained by targeted mutagenesis (Burchard et al., 2014), or synthetically manufactured materials (Chen et al., 2014). In genotyping assays for rare variants, i.e. TPMT*2 or TPMT*3B, heterozygous controls are prepared by mixing wild-type and mutant DNA fragments/clones in equal concentrations. The mixing ratio is usually determined by measuring the optical density or fluorescence of intercalating dyes. However, the imprecision of these methods can create a disproportionate amount of wild-type and mutant DNA molecules in the mixture. Moreover, the ratios are only valid for the tested batches of RMs.

The aim of this study is to present a novel approach for preparing triple-compound heterozygous reference control material (TCH-RM) for TPMT genetic testing by using the technology of hybrid gene synthesis (Hughes et al., 2011) with the insertion of wild-type and mutant polynucleotide fragments into a single cloning vector.

**Material and Methods**

**Subjects**

We investigated 371 patients (132 men and 239 women, median age 45 years, range 18–84 years) at the Charles University Hospital in Hradec Kralove, who were suffering from inflammatory bowel disease, autoimmune hepatitis, systemic lupus erythematosus, idiopathic inflammatory myopathies, multiple sclerosis or rheumatoid arthritis. Genotyping was performed with the informed consent of the patients prior to the first administration of thiopurine drugs. Genomic DNA was extracted from 200 μl of blood using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

**Preparation of TCH-RM**

The synthesized 2446 bp polynucleotide chain (GenScript, Piscataway, NJ) contained six TPMT gene segments in lengths sufficient for reliable laboratory testing. The segments were aligned in the following order (Fig. 1): TPMT 238G wild-type (segment I; 510 bp; NCBI reference sequence code NC_000006.12; positions 15560–15051), 460G wild-type (segment II; 292 bp; positions 10889–10598), 719A wild-type (segment III; 421 bp; positions 2535–2115), 238C mutant (segment IV; 510 bp; positions 15051–15560), 460A mutant (segment V; 292 bp; positions 10598–10889), and 719G mutant (segment VI; 421 bp; positions 2115–2535). The

![Fig. 1. Structure of a pUC57 vector containing six synthetic segments of the TPMT gene. Segments I and IV include a nucleotide sequence specific for TPMT 238G>C polymorphism; segments II and V for 460G>A; and segments III and VI for 719A>G. Segments I-III are wild-type sequences, segments IV-VI contain mutant sequences.](image-url)
complete chain was inserted into a pUC57 cloning vector; the constructs were transferred into an E. coli competent strain and amplified (GenScript). This was followed by purification, spectrophotometric determination of plasmid DNA concentration, and freeze-drying. After reconstituting the freeze-dried DNA molecules in Tris-EDTA buffer, serial dilution (10⁻¹⁰ copies/µl) in plastic tubes was performed. Aliquots of 20 µl diluted DNA were stored at -80 °C. TCH-RM characteristics were assessed via four various methods: reverse hybridization, real-time PCR with hydrolysis probes, real-time PCR followed by subsequent melting temperature analysis, and dideoxynucleotide DNA sequencing.

Reverse hybridization assay

TCH-RM at a concentration of 10° copies/µl was used for multiplex PCR with biotinylated primers and reverse hybridization on nitrocellulose strips according to the manufacturer’s instructions (PGX-TPMT StripAssay Kit, ViennaLab, Vienna, Austria). The lengths of the PCR products were 137 bp (TPMT*2), 165 bp (TPMT*3B), and 255 bp (TPMT*3C).

Real-time PCR assays

Real-time PCR with melting temperature analysis was performed in the LightCycler 2.0 (Roche Diagnostics, Penzberg, Germany) using LightSNiP hu TPMT*2 rs1800462, LightSNiP hu TPMT*3B rs1800460, and LightSNiP hu TPMT*3C rs1142345 assays with minor modifications (Pozler et al., 2010) of the conditions specified by the manufacturer (TIB MolBiol, Berlin, Germany). Allelic discrimination assays used real-time PCR with fluorescent hydrolysis probes (Yi TPMT Kit, Ybux, Brno, Czech Republic) for c.238G>C, c.460G>A, and c.719A>G TPMT polymorphisms were carried out in a Rotorgene 6000 (Corbett Research, Sydney, Australia) following the manufacturer’s protocol.

DNA sequencing

PCR amplicons were generated in mixtures (25 µl) containing 10× concentrated PCR buffer, 200 mM each of deoxynucleotides, 400 nM primers (Generi Biotech, Hradec Kralové, Czech Republic), 2.5 mM magnesium chloride, 50 ng of DNA, and one unit of Taq polymerase HS (Takara, Shiga, Japan). The following sequences of forward and reverse primers were used: 5'- CCT GCA TGT TCT TTG AAA CCC TAT GAA -3' and 5'- GCT TGA GTA CAG AGA GGC TTT GAC CTC -3' for TPMT*2; 5'- CTC CAC ACC CAG GTC GAC ACA TT -3' and 5'- GTA TAG TAT ACT AAA AAA TTA AGA CAG CTA AAC -3' for TPMT*3B; and 5'- AAT CCC TGA TGT CAT TCT TCA TAG T -3' and 5'- CAC ATC ATA ATC TCC TTC CTT CC -3' for TPMT*3C analysis. After initial denaturation (5 min at 95 °C), PCRs were run for 35 cycles consisting of 40 s denaturation at 95 °C, 40 s annealing at 55 °C, and 60 s extension at 72 °C in the ABI 2720 thermal cycler (Life Technologies, Carlsbad, CA). The sizes of the amplicons for c.238G>C, c.460G>A, and c.719A>G were 510 bp, 292 bp, and 421 bp, respectively. The same primers were used for bidirectional sequencing with a BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI 3130 Genetic Analyzer (Life Technologies). Unincorporated dye terminators, salts, and unused primers were removed by the Biomek 4000 Automated Laboratory Workstation (Beckman Coulter, Fullerton, CA).

Testing TCH-RM stability

The storage stability of DNA molecules in the TCH-RM aliquots containing 10⁶ copies/µl was examined using real-time PCR (Yi TPMT Kit) under the same conditions as above. Differences in cycle thresholds (Ct) were evaluated after six months of storage. The storage period was interrupted six times by thawing the tubes to room temperature for 30 min.

Statistical analysis

All experiments were done in triplicates. Calculations of means, standard deviations, coefficients of variation (CV), and mean differences were performed using the Statistica software (version 11, StatSoft, Tulsa, OK) for Windows.

Results

The triple heterozygous character of TCH-RM was shown by the presence of six bands specific for wild-type and mutant TPMT alleles on the hybridization test strips (Fig. 2a). Their intensities corresponded well to the results from the heterozygous subjects (Figs. 2b, 2c).

The storage stability of DNA molecules in the TCH-RM triple-compound heterozygosity was further verified by dideoxynucleotide sequencing analysis. The electropherograms showed typical double peaks visible in all the polymorphic sites (Fig. 3).

The results of the melting temperature analysis of TCH-RM for c.238G>C, c.460G>A, and c.719A>G polymorphisms are presented in Fig. 4. Melting temperatures for the tested allelic variants were as follows: 54 °C for the 238G wild-type allele, 64 °C for 238C, 53 °C for 460A, and 719A>G for 719A.

Fig. 2. Reverse hybridization analysis of the TPMT gene. Strip a: synthetic-triple-compound heterozygous reference material; strip b: 238G>C heterozygous patient (genotype *2/*1); strip c: 460G>A and 719A>G heterozygous patient (genotype *3A/*1).
Also, allelic discrimination real-time PCR confirmed its triple heterozygous character and a high degree of commutability in the range of five concentration orders (from $10^{2}$ to $10^{6}$ copies/µl), as demonstrated in Fig. 5.

Table 1 indicates Ct values of triplicates observed in allelic discrimination real-time PCR. The differences between the mutant and wild-type TPMT fragments of all three studied polymorphisms were less than 1.1 cycle if a threshold of 0.05 was chosen. When we evaluated the stability of the frozen TCH-RM aliquots via real-time PCR quantification, we achieved highly concordant triplicate data with coefficients of variability lower than 4% for all three assays. The differences in mean Ct values ($\Delta$Ct) before and after six months of storage were lower than one amplification cycle.

The determined genotypes of the patients were as follows: 352 wild-type subjects (94.8%), 17 TPMT*3A heterozygotes (4.6%), one patient heterozygous for the TPMT*2 allele (0.3%), and one TPMT*3C heterozygote (0.3%). No other TPMT genotype was found. The frequencies of TPMT*1, *3A, *3C, and *2 in the entire cohort were 97.5 %, 2.3 %, 0.1 %, and 0.1 %, respectively.

**Discussion**

TPMT genotyping is one of the most basic pharmacogenetic markers. Results of the analysis must be available prior to starting thiopurine drug therapy, and for such analysis simple, rapid, and reliable assays based on real-time PCR are currently preferred (Roman et al., 2012; Burchard et al., 2014; Chen et al., 2014).

Each experimental run performed in clinical labs should include control samples whose results support the validity of the analytical processes used. Homozygous controls are usually made by mixing either wild-type or mutant DNA fragments associated with the polymorphic sites tested. However, preparing a multi-allelic heterozygous RM for molecular diagnostics is made complicated by i) discrepancies in amplification efficiencies between site-specific wild-type and mutant DNA frag-
ments, ii) the varying quality, purity, and stability of the fragments, and iii) imprecision in determining the concentrations of wild-type and mutant fragments in the final mixture.

We suggest a unique RM consisting of six polynucleotide segments forming a TPMT 238G>C/460G>A/719A>G triple-compound heterozygous genotype. Due to the almost complete homogeneity of the appropriate pairs of wild-type and mutant segments differing in one nucleotide only, assembling them into direct tandems during the synthesis was impossible (Beranek et al., 2014). Therefore, we used an alignment of the segments according to the absence/presence of the mutation: the first three segments contained parts of the coding sequence of the TPMT*1 close to c.238G>C, c.460G>A, and c.719A>G polymorphic sites; the second three contained the nucleotide sequences of the TPMT*2, *3B, and *3C allelic variants.

The lengths of the segments were selected to reach a high degree of commutability for the various analytical techniques. Similar ranges of synthetic polynucleotides are currently used when preparing control materials for TPMT genotyping in a traditional manner (Burchard et al., 2014).

The nucleotide sequences within the segments contained several other polynucleotides that only rarely influence the enzymatic activity of TPMT in the population (Zhou and Chow, 2007): *3D (G292T) and *19 (A365C) in segments I and III; *10 (G430C), *15 (IVS-1G>A), *16 (G488A), and *22 (G488C) in segments II and IV; and *7 (T681G) and *8 (G644A) in segments III and VI. The sequencing analysis performed was able to confirm the wild-type character of TCH-RM in these sites.

All the testing methods yielded concordant results of TCH-RM analysis, thus showing its compound heterozygosity, universality, and commutability. Concentrations of TCH-RM ranging from 10^4 to 10^6 copies/µl provided amplification curves comparable with those obtained for human DNA extracted from blood or buccal swabs. No signs of degradation during the storage of TCH-RM at -80 °C were recorded. Therefore, the properties of our multi-allelic RM support its availability for real-time PCR amplification, especially if the TPMT polymorphisms are tested simultaneously in one experimental run.

Using TCH-RM, we identified 19 TPMT heterozygotes in the group of patients. The frequencies of TPMT genotypes and allelic variants were in agreement with previous studies performed on Caucasians (Ameyaw et al., 1999; Haglund et al., 2004; Gearry and Barclay, 2005; Almoguera et al., 2014). All the heterozygotes expressing intermediate TPMT activity were treated with thiopurines, a process that started with a reduced dose and increased incrementally to a target weight-based dose according to recent guidelines (Relling et al., 2013; MacDermott, 2015).

In conclusion, assembling segments of synthetic DNA into long polynucleotide chains is a universal way of obtaining compound heterozygous material that can then be used to perform any simultaneous analysis of polymorphic sites in the human genome. The batches are manufactured with a perfect concentration match of wild-type and mutant fragments, and can be made in large quantities and in lengths suitable for most diagnostic techniques.

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