

Implementation of TPMT testing

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The activity of the enzyme thiopurine methyltransferase (TPMT) is regulated by a common genetic polymorphism. One in 300 individuals lack enzyme activity and 11% are heterozygous for a variant low activity allele and have an intermediate activity. The thiopurine drugs azathioprine, mercaptopurine and thioguanine are substrates for TPMT; these drugs exhibit well documented myelosuppressive effects on haematopoietic cells and have a track record of idiosyncratic drug reactions. The development of severe bone marrow toxicity, in patients taking standard doses of thiopurine drugs, is associated with TPMT deficiency whilst the TPMT heterozygote is at an increased risk of developing myelosuppression. Factors influencing TPMT enzyme activity, as measured in the surrogate red blood cell, are discussed in this review to enable an appreciation of why concordance between TPMT genotype and phenotype is not 100%. This is particularly important for lower/intermediate TPMT activities to avoid misclassification of TPMT status. TPMT testing is now widely available in routine service laboratories. The British National Formulary suggests TPMT testing before starting thiopurine drugs. Dermatologists were quick to adopt routine TPMT testing whilst gastroenterologists do not specifically recommend TPMT screening. TPMT testing is mandatory prior to the use of mercaptopurine in childhood leukaemia. Thiopurine drug dose and other treatment related influences on cell counts explain some of the differing recommendations between clinical specialities. TPMT testing is cost-effective and the major role is in the identification of the TPMT deficient individual prior to the start of thiopurine drugs.

Introduction

Individual variations in human red blood cell (RBC) thiopurine methyltransferase (TPMT, E.C.2.1.1.67) activity were first described by Weinshilboum *et al.* [1] in the late 1970s. Subsequent Caucasian population studies demonstrated that the level of TPMT activity was inherited in an autosomal codominant fashion. The frequency distribution of TPMT activities conformed to Hardy–Weinberg predictions for the inheritance of two alleles one for high ($TPMT^H$) and one for low ($TPMT^L$) enzyme activity. Approximately 89% of a randomly selected population were homozygous for an allele for high RBC TPMT activity, about 11% heterozygous with an intermediate activity and one in every 300 subjects homozygous for an allele for low RBC TPMT activity, the latter lacking detectable TPMT activity [2]. It was soon established that the genetic polymorphism

controlling RBC TPMT activity also controlled the level of enzyme activity in all other cells and tissues [3–5] but it was over a decade later before the *TPMT* gene was isolated, sequenced and the variant alleles described at a genetic level [6–8]. Controversy remains over various aspects of TPMT genotype/phenotype concordance and whether genotype or phenotype is the most accurate predictor of TPMT status.

The TPMT genetic polymorphism represents a well validated example of the clinical importance of pharmacogenetics [9]. Very low, or deficient, TPMT activity is associated with grossly abnormal thiopurine drug metabolism, excess production of cytotoxic metabolites and profound life-threatening myelotoxicity, in patients taking thiopurine drugs. Although this association was reported in the late 1980s [10], there was, initially, a minimal use of TPMT testing prior to the start of thiopurine

Mercaptopurine pharmacogenetics were exposed to a wider clinical audience when the clinical importance of the individualization of thiopurine therapy, underpinned by the TPMT genetic polymorphism, emerged as relevant to thiopurine immunosuppression in, primarily, inflammatory bowel disease (IBD) [37, 38]. Red cell TGNs were associated with clinical response to mercaptopurine in paediatric and adult IBD [39–41]; TPMT heterozygotes accumulated higher TGN concentrations [39]. IBD patients with lower TPMT activities had a statistically significant relapse free advantage [42]. However, all adverse events (e.g. myelotoxic, gastrointestinal, allergic), were significantly more frequent in IBD patients with lower TPMT activities than those with 'normal' activity [43, 44]. The narrow therapeutic window for mercaptopurine was confirmed and both disease control and adverse events were associated with higher TGN concentrations [39–41, 43, 44]. Thiopurine testing improved the clinical outcome [45]. However, it was clear that adverse events were not purely regulated by TPMT. Other factors potentiate these events, but those with lower TPMT activities are at a significantly increased risk of suffering these events.

The TPMT enzyme

TPMT is a cytoplasmic enzyme which catalyzes the S-methylation of aromatic and heterocyclic sulphhydryl compounds, but the endogenous function of TPMT is unknown. The catalytic specificity of TPMT was investigated by Weinshilboum's group in the 1990s. Substrates include thiopurine drugs, selenium compounds and the disulfiram metabolite, diethyldithiocarbamate [46, 47]. Both mercaptopurine and thioguanine (INN, tioguanine) are good substrates for the enzyme and, of their nucleotide metabolites, mercaptopurine nucleotide (thioinosine monophosphate) is a far better substrate than the TGNs. The latter are very poor substrates [48] but are methylated *in vivo* at concentrations over 500 pmol/ 8×10^8 red cells [49]. Oxidation of the thiopurine ring (via xanthine oxidase, EC 1.11.3.22) produces 8-hydroxymercaptopurine, a good TPMT substrate and the 2-hydroxy products, thioxanthine (2-hydroxymercaptopurine) and thiouric acid (2,8-hydroxymercaptopurine). Both are potent TPMT inhibitors (Figure 1). At very high concentrations (K_i 0.56 mM) the TPMT product methylmercaptopurine also inhibits human kidney TPMT [48]. This raises the possibility that, *in vivo*, thiopurine metabolism may modulate TPMT activity.

TPMT is inhibited by many benzoic acid derivatives. Perhaps the most important are the aspirin metabolite salicylic acid and 5-aminosalicylate medications such as sulphasalazine and mesalazine [50]. The latter is widely used in the treatment of IBD, and other immunosuppressive disorders, for which mercaptopurine or azathioprine may be co-administered and drug–drug interactions have been reported. Inhibited TPMT results in the production of

elevated TGN concentrations and an increased risk of myelotoxicity [51–53]. However, such TPMT inhibition would not be reflected in *in vitro* measurements of enzyme activity [54].

Measurement of TPMT status

Analytical methods for the measurement of TPMT genotype and phenotypic activity, initially developed in academic medical centres, have been adapted for the routine service sector and a growing number of thiopurine monitoring and TPMT screening services are now available [55–60].

TPMT phenotypic activity

TPMT phenotype is measured in the RBC by radiochemical [1] or chromatographic techniques [57, 61–64]. These activities are influenced by red cell transfusions. Additionally, in children with ALL, there are disease [65] and treatment-related [27] influences on TPMT activity. For the interpretation of TPMT phenotype, enzyme activities must be measured under constant conditions at a standardized dosage during a specific phase of treatment. These leukaemia- and treatment-related changes do not occur in IBD. TPMT activities measured prior to, and during, thiopurine treatment are the same [66, 67]. Reporting phenotype in terms of haemoglobin concentration, packed red cells, erythrocyte counts or protein content give similar accuracy and power to predict heterozygous and wild-type individuals [68].

Shifts in the TPMT activity frequency distribution have been widely reported by groups investigating phenotypic activities. These 'shifts' are at the most pronounced in children with ALL when the frequency distribution is shifted downwards, well below the range recorded for healthy children, at disease diagnosis, [27, 69] whilst chemotherapy the distribution shifts upwards, well above the range recorded for healthy children [27, 69, 70]. Other disease-dependent fluctuations in TPMT activity distributions are reported to be clinically insignificant [71]. In healthy populations, children are reported to have higher TPMT activities than adults [72], younger children higher activities than older children [72] with healthy neonates having the highest activities [73]. These shifts are probably influenced by the age profiles of the red cell population, younger red cells having higher TPMT activities than older cells [65]. Pancytopenia markedly elevates measured red cell TPMT activities [74].

A gender difference has been reported in the distribution of red cell TPMT activities in some [75, 76] but not all studies [2, 36], with wild-type TPMT infants less than 2 years having a higher activity in boys than girls [72]. Hepatic activity (measured in patient surgical biopsy tissue) was significantly higher in men than women; but this difference was not reflected in measurements of red

cell TPMT activity [5]. In addition, TPMT phenotypic measurements show an interethnic variability. Early studies reported that white Caucasian populations have higher activities than North American Black populations whilst indigenous Norwegian Saami populations have higher activities than Caucasians [77–79]. However the TPMT activities measured were similarly polymorphic with a trimodal distribution reported in all three populations. This is not so in Asian populations. Singapore Malay have higher activities than either Singapore ethnic Chinese or Indian populations. The distribution of TPMT activities is binomial in all these populations [80, 81] whilst unimodal, normal, distributions are reported in healthy Korean populations [82]. These latter reports, emanating from a number of different laboratories but also from studies of diverse populations within the same laboratory, could not be attributed to differences in enzyme activity methodologies. A major factor in these ethnic differences was the inheritance of specific TPMT low activity variant alleles within these populations [83–85]. Thus, ethnicity, type of disease, concurrent drug treatment and red cell kinetics and transfusions must be considered when interpreting TPMT activity measurements.

TPMT genotype

Single nucleotide polymorphisms (SNPs) account for the major TPMT low activity variant forms [6, 8, 9, 57, 64]. Genotyping for the TPMT*3 family of variant alleles will detect over 92% of low activity alleles, inclusion of TPMT*2 pushes this to over 95% [9, 57, 64]. Whilst TPMT*3A is the most common variant allele in Caucasian populations [8, 36, 64] TPMT*3C is the common mutant allele in African populations [84] with the African-American population having an approximate 50:50 mix of TPMT*3A and TPMT*3C [86]. In these populations the variant allele frequency is about 5%. This is in contrast with a frequency of 1 to 3% reported in Asian populations when the dominant allele is TPMT*3C [80, 83, 87]. Over 35 variant low activity TPMT alleles have been reported and, to avoid a duplication of allele numbering, a specific logical nomenclature system has been adopted, for the designation of novel allele names in humans, which is maintained and managed by the TPMT Nomenclature Committee [87, 88]. The incidence of rare or novel alleles in Caucasian populations has been estimated to be approximately 1 in 200 [64, 87, 88].

TPMT genotype–phenotype concordance

The overall concordance between genotype and phenotype in healthy volunteers is 98.4%, but in the ‘intermediate’ range of TPMT activities this falls to 86%. Approximately 1 in 20 could have novel mutations but 1.6% of individuals with intermediate activities are wild-type in open reading frame [64]. A recent systematic review of TPMT testing in adults and children with chronic inflammatory diseases concluded that genotyping specificity

approached 100%, but the ability of genotype to identify patients with an intermediate activity was imprecise, ranging from a pooled estimate of 70% to 86% [89]. As previously discussed, there are many possible treatment-related, environmental and ethnic influences on phenotypic TPMT activity that could contribute to lack of concordance in the intermediate activity range. In addition, modulation of TPMT activity by tandem repeats within the TPMT promoter has been proposed [90], but larger population studies have shown these effects to be quantitatively small [91].

Neither TPMT genotype nor phenotype alone can be 100% guaranteed to identify the TPMT deficient individual. Genotype tests for the TPMT*3 family and TPMT*2 cover 95% of inactivating alleles and phenotype tests can be used to double-check the TPMT heterozygote for the estimated 1 in 7416 chance of TPMT deficiency due to a rare/novel variant allele [92, 93]. However, when using phenotypic enzyme activity, as the initial TPMT test, the risk of misclassifying a TPMT deficient patient as one with intermediate activity is higher than the risk of missing the deficient patient by TPMT genotype [93]. A comparison of TPMT testing methods in a National Centre showed genotype to be superior to phenotype. Using the phenotype assay 11% of TPMT deficient individuals would have been misclassified as TPMT intermediate activity due to TPMT enzyme activities above the TPMT deficient cut-off value when the TPMT activity was measured in the laboratory under ideal conditions. TPMT genotype was recommended as the primary test [93]. Test centres employing phenotypic methodologies frequently use TPMT genotype as an assurance tool to check the intermediate activity cohort for the true positives [92]. Although some laboratory forms for the TPMT test request information on prior blood transfusions, it was not stated if this was so for the test centre results previously discussed [92, 93] and this could be a major contributory factor for the observed TPMT discordance, particularly for the TPMT deficient phenotype. However, in a study using blood samples taken prior to any transfusions, the accuracy of TPMT genotype was far superior to phenotypic activity measurements in leukaemia patients, a clinical situation in which the latter test is unreliable [36, 94].

It has been argued that TPMT genotype analysis will not, as yet, provide that additional information yielded by phenotype, i.e. the identification of those with very high activities who may require escalated dosages to produce an adequate clinical response and those with wild-type genotype with functional ‘intermediate’ activity. However, the latter group mainly consists of false positives produced by variable red cell kinetics due to the underlying disease state or other factors [36] whilst in the former, the preferential production of methylated mercaptopurine metabolites (products of the TPMT reaction) and suboptimal response to thiopurine therapy is not always attributed to very high TPMT activities [95]. Frequently, the search for

additional information to interpret the clinical picture requires thiopurine metabolite analysis in addition to TPMT status. TPMT testing is a predictor of what could happen during thiopurine therapy, metabolite monitoring is one step nearer the pharmacological action and the latter is a useful adjunct to TPMT status information when compliance with oral therapy is suspected as the cause of drug tolerance [36] or when myelosuppression may be attributed to multiple causes or other toxicity symptoms require investigation.

Cost effectiveness of TPMT testing

Because of life threatening nature of thiopurine drug-related toxicity, prospective identification of patients with TPMT deficiency (homozygous for variant low activity TPMT alleles), prior to the initiation of therapy has increasingly been accepted clinically. The cost of in-patient care for one TPMT deficient patient inadvertently treated with azathioprine has been estimated to cover the cost of over 400 tests for TPMT activity [96]. The cost of TPMT testing is offset by the improved patient care and the improved quality of life for the TPMT deficient patient. The cost effectiveness of TPMT screening prior to mercaptopurine or thiopurine drug therapy has been repeatedly stated [45, 88, 97, 98].

Recommendations for, and use of, TPMT testing

The US Federal Drug Administration directed label modifications for 6-mercaptopurine (July 2004) and azathioprine (July 2005) to reflect the pharmacogenetics of thiopurine metabolism and recommend TPMT testing prior to the initiation of thiopurine therapy [9]. TPMT testing prior to the prescription of thiopurine drugs is becoming routine clinical practice in Europe [12–15]. In the UK, a recent survey indicated that TPMT testing was used by 67% of clinicians prior to azathioprine prescription [99] whilst, worldwide, testing is used by 43% of gastroenterologists in the management of IBD [100]. Overall, UK clinical guidelines recommend that patients have their TPMT status checked prior to starting thiopurine drugs. In the UK TPMT testing is mandatory for children and young adults prior to treatment on the ALL2011 trial protocol [101].

A systematic review of TPMT testing in adults and children with chronic inflammatory diseases reported that, compared with a wild-type TPMT genotype, those who were TPMT heterozygotes or TPMT deficient were at an increased risk of developing leucopenia (odds ratios 4.29, 95% CI 2.67, 6.89 and 20.84, 95% CI 3.42, 126.89, respectively). However, there was insufficient evidence to address the effectiveness of TPMT pre-testing with respect to improved patient outcomes [89]. A similar meta-analysis

of 67 studies reported an odds ratio of 4.06 (95% CI 3.2, 5.48) for intermediate activity patients developing leucopenia compared with patients with high (or 'normal') TPMT activity when taking thiopurine medication; 86% of patients with two variant alleles developed myelosuppression [102].

Guidelines to interpret TPMT genotype tests in order to guide the dosing of thiopurines have been developed [14, 15]. Patients who inherit two variant low activity alleles (TPMT deficiency) will, with 100% certainty, develop life-threatening myelosuppression at conventional thiopurine dosages; drastic dose reduction (e.g. 10% of conventional dose) is required. About 30 to 60% of TPMT heterozygotes will be unable to tolerate full thiopurine doses [14, 21, 36]; TPMT heterozygotes are at a significantly higher risk for toxicity than TPMT wild-type patients [102]. Thus a reduced thiopurine dose for the TPMT heterozygote has been recommended at the start of treatment [103]. The latter approach risks the under-dosing of those TPMT heterozygous patients who can tolerate full doses, and so a titration upwards approach is advised [14].

The British National Formulary suggests that the clinician should consider measuring TPMT activity before starting thiopurine drugs [104]. An initial assessment of TPMT testing in the UK [12] reported that the test was requested by 13 different medical specialities with dermatologists and gastroenterologists the most frequent users. A more recent survey reported that 94% of dermatologists and 60% of gastroenterologists requested TPMT testing [99]. The high uptake by dermatologists is reflective of the fact that they were the first speciality in the UK to develop national guidelines advocating the use of TPMT testing [105]. Current guidelines for dermatologists review the case for TPMT testing and firmly support routine pre-treatment TPMT testing and emphasize the cost-effectiveness against the intensive support care required for patients with severe and prolonged myelosuppression [106]. The guidelines for rheumatologists recommend TPMT testing prior to prescribing azathioprine with the caveat that testing does not replace routine monitoring (of blood cell counts) [107].

In the treatment of autoimmune hepatitis (AIH), where long term azathioprine is the immunosuppressive of choice for the maintenance of remission and is used with prednisolone to induce remission, the UK guidelines state that TPMT measurement 'should be considered' to exclude those with TPMT deficiency and TPMT measurement 'is recommended' in patients with pre-existing leucopenia [108]. The USA AIH guidelines state that the frequency of cytopenia is 46% in azathioprine treated AIH patients but studies to date have shown that this is not predicted by prior knowledge of TPMT status. The most common cause of cytopenia in the AIH patient is hypersplenism associated with underlying cirrhosis. The USA guidelines comment that TPMT deficiency is rare and the azathioprine dose used in conventional treatment is low. Thus

they do not support routine TPMT screening but the USA guidelines recommend that TPMT activity should be assessed in patients with cytopenias before or during azathioprine therapy [109].

UK gastroenterologists view TPMT testing with caution, initially not recommending prior to therapy on the basis that decades of experience had shown azathioprine to be a safe drug in the treatment of inflammatory bowel disease [110]. The current British Society of Gastroenterology guidelines acknowledge the role of TPMT testing in identifying the 1 in 300 TPMT deficient patient and comment that most patients who develop leucopenia will have a 'normal' (high activity, homozygous wild-type genotype) TPMT. TPMT testing is not specifically recommended, although a role is suggested of predicting early events rather than long term control [111]. Nonetheless, gastroenterologists are among the most frequent users of TPMT testing in the UK [99].

Despite the widespread use of TPMT testing debate continues with respect to the utility of TPMT testing [112]. With a lack of prospective randomized controlled trials the evidence base for routine TPMT testing remains suboptimal [113]. The UK Department of Health funded TARGET (TPMT: Azathioprine Response to Genotyping and Enzyme Testing) controlled trial investigated the clinical value and cost-effectiveness of TPMT genotyping in reducing the number of adverse drug reactions associated with azathioprine immunosuppression [114]. The recruitment target ($n = 500$) was not met due to the routine use of TPMT testing in some treatment centres. The study ($n = 333$) concluded that there was strong evidence for severe neutropenia in the TPMT deficient patient, but TPMT heterozygotes were not at an increased risk of adverse drug reactions at standard doses of azathioprine.

What is clear is that adverse reactions to thiopurine drugs are dose dependent and, of the myriad of adverse reactions attributed to the thiopurine drugs, myelosuppression is more common when thiopurine drugs are used more aggressively, as in the 6-mercaptopurine treatment protocols for childhood ALL, than when used in low dose azathioprine immunosuppressive therapy. In addition, concomitant therapy and the underlying disease process can also influence the susceptibility to myelosuppressive events for all patients. However, unless treated on very low thiopurine doses (in ALL protocols 10%, or less, of the standard protocol mercaptopurine doses are used) the TPMT deficient patient will experience profound myelosuppression when treated with thiopurine drugs. It is cost-effective to routinely perform pre-treatment TPMT testing to identify these individuals alone.

Competing Interests

The author has completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (avail-

able from request from the corresponding author) and declares support from Leukaemia and Lymphoma Research for the submitted work, no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years and no other relationships or activities that could appear to have influenced the submitted work.

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