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GENOTYPING SHOULD BE CONSIDERED THE PRIMARY CHOICE FOR
PRE-TREATMENT EVALUATION OF
THIOPURINE METHYLTRANSFERASE FUNCTION

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Running Head: Genotyping as pre-treatment evaluation of TPMT function

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genotype; phenotype

Abstract

Background and aims: A pre-treatment determination of the thiopurine S-methyltransferase (TPMT) genotype or phenotype can identify patients at risk of developing severe adverse reactions from thiopurine treatment. The risk of misclassifying a patient might be dependent on the method used. The aim of this study was to investigate the concordance between TPMT genotyping and phenotyping.

Methods: The data consist of 7195 unselected and consecutive TPMT genotype and phenotype determinations sent to the division of Clinical Pharmacology, Linköping, Sweden. TPMT activity was measured in red blood cells (RBC) and the genotype determined by pyrosequencing for the three most common TPMT variants (TPMT *2, *3A, *3C).

Results: TPMT genotyping identified 89% as TPMT wild type (*1/*1), 10% as TPMT heterozygous and 0.5% as TPMT defective. The overall concordance between genotyping and phenotyping was 95%, while it was 96% among IBD patients (n=4024). Genotyping would have misclassified 8% of the TPMT defectives as heterozygous as compared to 11% if only TPMT activity had been measured. Eleven percent of the heterozygous patients had a normal TPMT activity (>8.9 U/ml RBC) and 3% of the TPMT wild-type patients had an intermediate TPMT activity (2.5–8.9 U/ml RBC).

Conclusions: There is a risk for TPMT misclassification when only genotyping or phenotyping is used, but it is not reasonable to check both in all patients. Since TPMT genotyping is the more reliable test, especially in TPMT heterozygotes, we suggest that genotyping should be considered the primary choice for the pre-treatment evaluation of TPMT function before initiation of thiopurine therapy.

1. Introduction

In the treatment of inflammatory bowel disease (IBD) a substantial part of the patients are qualified for long-term immunomodulation. The thiopurine drugs azathioprine (AZA) and 6-mercaptopurine (6-MP) are the mainstay in this respect and they are increasingly and more liberally used in IBD (1). Thiopurine S-methyltransferase (TPMT) is a key enzyme in the metabolism of thiopurine drugs and genetic polymorphisms in the TPMT gene are associated with decreased TPMT activity and the development of myelotoxicity due to high TGN metabolite concentrations (1).

The main purpose of TPMT screening is to identify the TPMT defective patients at risk of developing severe bone marrow suppression (2). This can easily be done by obtaining a pretreatment determination of either the TPMT phenotype or genotype. The risk of misclassifying a TPMT defective patient as a TPMT heterozygote might, however, be dependent on the method used.

A secondary purpose of TPMT screening is to find the TPMT heterozygote patients, since they are at intermediate risk of bone marrow toxicity (3) and have an increased risk of treatment discontinuation if put on a normal thiopurine dose (4, 5).

The knowledge about the TPMT gene is constantly evolving and new inactivating mutations associated with decreased TPMT activity are described on a regular basis. Currently, at least 31 such variant alleles have been reported in humans (TPMT *2 to *28) (1, 4-8). This will have consequences on the interpretation of standard TPMT genotypic analyses, which generally only determine the most common TPMT alleles.

The TPMT phenotype has a trimodal frequency distribution in Caucasian populations of healthy blood donors with distinct TPMT activity cut-off levels between the three phenotypes (9). In a perfect scenario, a low TPMT activity should be observed in all TPMT defective patients, while an intermediate TPMT activity should be seen among all TPMT heterozygotes

and a normal TPMT activity among all TPMT wildtype individuals. However, the TPMT phenotype is dynamic and is affected by both inter- and intraindividual variations in all current methods used for TPMT activity determination. Furthermore, a number of pre-analytical factors, like blood transfusions (2) and an altered hematopoiesis (10), are well known to affect the TPMT activity. All these factors may lead to a TPMT phenotype misclassification.

Despite the above mentioned limitations of both tests, the TPMT genotype-phenotype concordance reported in the literature is generally very high (13). It has, however, not been assessed in a large unselected patient population. The aim of this study was to investigate the correlation between TPMT genotype and phenotype in a large unselected patient population and analyze the results from a clinical and practical perspective.

2. Methods

The data from this study consist of unselected and consecutive TPMT phenotype and genotype determinations sent to the division of Clinical Pharmacology, Linköping University, Sweden between 2006 and 2010.

2.1 TPMT enzyme activity measurement

Routine phenotyping was performed according to a previously described radiochemical method (14). In short, TPMT activity was measured in red blood cells from quantification of ¹⁴C-methyl-mercaptopurine produced by TPMT from 6-mercaptopurine with S-adenosyl-L-

¹⁴C-methyl-methionine as the methyl donor. Enzyme activity was expressed as units (U) TPMT per ml of packed red blood cells (pRBC) per hour of incubation. In TPMT activity measurements, we use the terms low, intermediate and normal TPMT activity. To distinguish between low, intermediate, and normal enzyme activity, cutoff levels of 2.5 and 9.0 U/ml pRBC, respectively, were used.

2.2 TPMT genotyping

DNA was isolated from whole blood using the QIAamp[®] DNA Blood Mini Kit (Qiagen, Hilden, Germany). The TPMT genotype was determined by a pyrosequencing method as described previously by our group (11). The patients were genotyped for 238 G>C, 460 G>A and 719 A>G polymorphisms (TPMT*2, *3A and *3C). Individuals that had a TPMT enzyme activity below 9.0 U/ml pRBC, and who were wild type at positions 238, 460 and 719, was further investigated by capillary sequencing of TPMT exon III to X as earlier described (10). An individual with two non-functional alleles is considered to be TPMT defective, while an individual with one non-functional allele is considered to be TPMT heterozygote and an individual with two functional alleles is defined as having a normal TPMT genotype (wild type).

2.3 Statistical analysis

In context of concordance between TPMT geno and phenotyping, column proportions were compared using the column proportions test with post hoc bonferroni correction using IBM SPSS statistics 19. A p-value <0.05 was considered to be significant.

2.4 Ethical considerations

The data presented in this study are the results from a quality assurance audit of the TPMT database at the Clinical Pharmacology Laboratory at Linköping University. This audit was designed to retrospectively evaluate the concordance between the TPMT genotypic and phenotypic analyses performed at the laboratory with the aim of improving our clinical service. Results have only been studied on a group level. A formal ethical approval is not necessary for data obtained during quality assurance audits according to Swedish national research ethics guidelines.

3. Results

3.1 Entire cohort

The TPMT activity and genotype were determined in 7195 individuals; 89% were TPMT *1/*1 (wild type), 10% were heterozygous for one non-functional allele and 0.5% carried two non-functional alleles (TPMT defective). The overall concordance between genotype and phenotype was 95%. The frequency distribution of TPMT activity was bimodal (Figure 1) and no distinct cut-off level between intermediate and normal TPMT activity was observed.

At least eight percent of the TPMT defective patients (3/37) would have been misclassified as heterozygotes with a standard genotyping screening for the three most common defective alleles. Further genotyping investigation detected uncommon defective alleles in these 3 individuals with a heterozygote TPMT genotype but with a low TPMT activity; TPMT *3A/*14 (n=1), *3A/*15 (n=1), and *3A/*23 (n=1). On the other hand, the TPMT phenotype would have misclassified in 11% (4/37) of the patients with a defective TPMT genotype due to results above the cut-off level for low TPMT activity (<2.5 U/ml RBC).

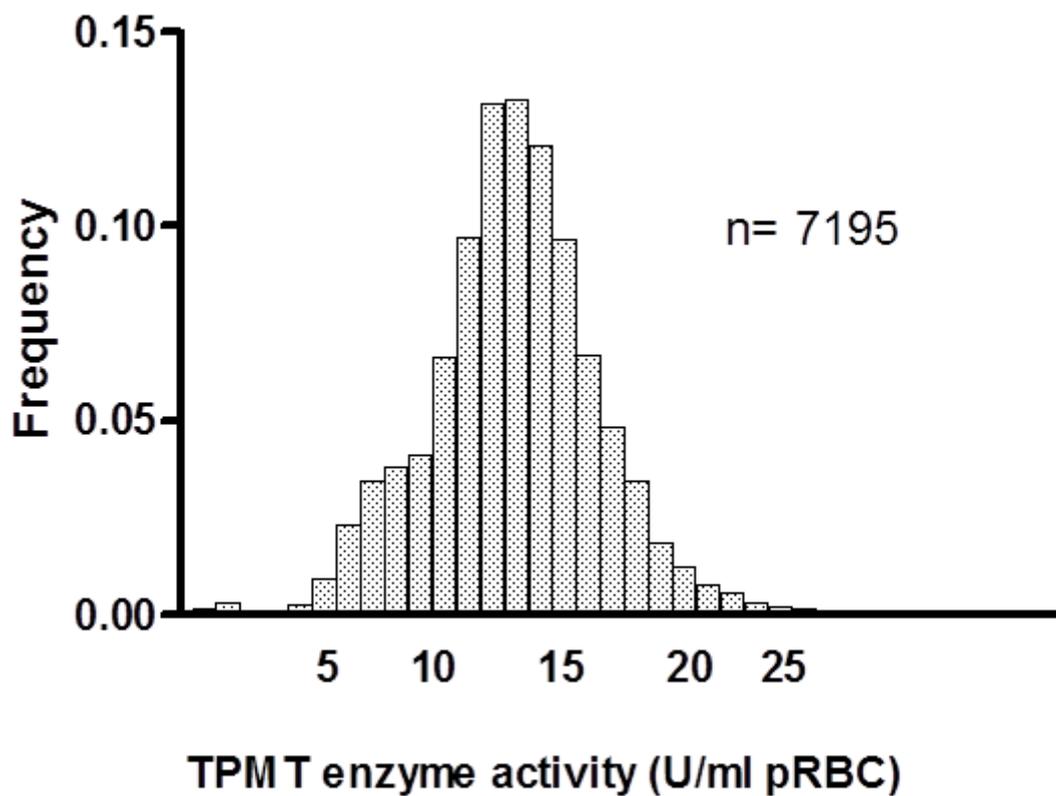


Figure 1.

TPMT frequency distribution in 7195 individuals. The data consist of unselected and consecutive TPMT phenotype and genotype determinations sent to the division of Clinical Pharmacology, Linköping University, Sweden between 2006 and 2010.

Overall, 291 TPMT wild type individuals (4.5%) had a TPMT activity lower than expected from genotyping, but 124 of these individuals (43%) had a haematological disorder (mainly acute lymphoblastic leukaemia (ALL)). Furthermore, 78 TPMT heterozygous individuals (11%) had a normal TPMT activity.

Six of the 704 TPMT heterozygote individuals (1%) had uncommon defective alleles not detected by a standard genotyping assay; TPMT *1/*3B (n=1), *1/*9 (n=1), *1/*14 (n=1), *1/*23 (n=2) and *1/*28 (n=1).

3.2 IBD patients

The genotype/phenotype concordance among IBD patients (n=4024) was 96%, but 11% of the TPMT heterozygotes had a normal TPMT activity (>8.9 U/ml RBC). However, only 3% of the TPMT wild-type IBD patients had an intermediate TPMT activity (2.5–8.9 U/ml RBC) compared to 31% of the hematological patients (n=446), in whom a disturbed haematopoiesis play a significant role (p<0.05).

4. Discussion

This study describes a large unselected sample of simultaneous TPMT phenotype and genotype determinations that represent what is actually seen at a laboratory in daily clinical practice and thus reflect a real-life situation. In this cohort the classical trimodal frequency distribution of TPMT activity was not seen.

The cut-off levels used to distinguish between the three TPMT phenotypes are based on smaller samples that generally are obtained from blood donors (9, 12). In a large unselected sample, like the one presented here, these cut-off levels are imperfect and a substantial part of the patients will be misclassified by the TPMT phenotype. This is of clinical significance,

since this population is at risk of dose-dependent thiopurine toxicity alternatively treatment with sub-therapeutic thiopurine doses.

The main purpose of TPMT screening is to identify the TPMT defective patients at risk of developing severe bone marrow suppression (2). A large population is needed to evaluate the absolute risk for misclassification of TPMT defective individuals given their rare occurrence and the even more unusual finding of new defective TPMT alleles in this group. In this study we observed that 3/37 TPMT defective individuals would have been misclassified as TPMT heterozygotes with the use of a standard genotyping for the three most common defective alleles (TPMT *2, *3A, *3C). On the other hand, 4/37 TPMT defective individuals would have been misclassified as having an intermediate TPMT activity due to results above the cut-off level for low TPMT activity (<2.5 U/ml RBC).

Overall, 4.5% of the TPMT wild type individuals had a TPMT activity lower than expected from genotyping. This can to a large extent be explained by a disturbed haematopoiesis (10) since 43% of these individuals was diagnosed with haematological disorders like ALL. Some IBD patients with a normal TPMT genotype and lower than expected TPMT activity exhibit a significant TPMT induction when put on thiopurines (13). There might also be individuals with novel variant alleles, not detected by current genotyping methods, in this group. Individuals, with an intermediate TPMT activity despite a normal TPMT genotype, are at risk of treatment with sub-therapeutic thiopurine doses if their TPMT function is only assessed by the phenotype. This assumption is based on the fact that patients with a heterozygote TPMT genotype or intermediate TPMT activity are known to produce higher TGN metabolite levels (14) and consequently clinicians often reduce the thiopurine dose (15). Although a clear relationship between thiopurine dose and TGN levels have not been described (16, 17), one could speculate that a markedly reduced thiopurine dose in patients with a normal TPMT

genotype might lead to sub-therapeutic TGN levels individuals and consequently an ineffective treatment.

The other group, which consists of individuals with a higher TPMT activity than expected from genotyping, is interesting from a clinical point of view as patients with a heterozygous TPMT genotype are at increased risk of bone marrow toxicity (3) and treatment discontinuation (13, 18) if put on a normal dose of thiopurines. Individuals in this group, with a heterozygote TPMT genotype but a normal TPMT activity, are likely to be treated with normal doses of thiopurines if the TPMT status is only assessed by the phenotype and accordingly at risk of unfavourable treatment outcome. Although this group only constitutes about 1% of the total population, every tenth TPMT heterozygote will be at risk if their TPMT function is assessed only by a TPMT phenotype determination. It must, however, be stressed that it is unclear to what extent these individuals, with a normal TPMT activity but a heterozygote TPMT genotype, will tolerate a normal dose of thiopurines.

Albeit the overall good concordance between TPMT genotype and phenotype in this study, there are still substantial genotype/phenotype discrepancies. The only way to identify all of them is by checking both the TPMT genotype and phenotype, or to use a genotyping assay which allows for detection of unknown variants.

In daily practice, clinicians often have to choose one of these methods due to economic or logistic limitations. In the choice between these two tests, we would recommend TPMT genotyping since it seems to be comparable with TPMT phenotyping when it comes to misclassifying the TPMT defectives (8% vs. 11%) but superior when it comes to misclassifying the TPMT heterozygotes (1% vs. 11%). Furthermore, the TPMT genotype is less affected by pre-analytical factors and it is probably easier for clinicians to interpret given its ternary result distribution (wildtype, heterozygote and homozygote defective).

In conclusion, TPMT deficient and heterozygote patients are at risk of misclassification when only TPMT genotyping or phenotyping is used. However, it is not reasonable to check both genotype and phenotype in all patients. Since TPMT genotyping is the more reliable test, especially for patients with a heterozygote genotype, we suggest that this test should be considered the primary choice for the pre-treatment evaluation of TPMT status before initiation of thiopurine therapy.

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Table 1. Concordance between TPMT genotype and TPMT phenotype in the entire cohort (n= 7195).

		TPMT activity (U/ml RBC)		
		Low	Intermediate	Normal
		(<2.5)	(2.5-8.9)	(>8.9)
TPMT genotype	*variant/*variant	33	4	0
	*1/*variant	3	623	78
	*1/*1	0	291	6163

Group at risk of sub-therapeutic dosage and ineffective treatment

↓

Group at risk of dose-dependent thiopurine toxicity

Table 2. Schematic illustration of defective alleles that would have been missed with a standard genotyping test only testing for the three most common defective alleles (*2, *3A and *3C).

	Variant(s) is *2, *3A or *3C	Variant(s) is NOT *2, *3A or *3C	Total
TPMT *1/*variant (heterozygous) n (%)	698 (99%)	6 (1%)	704
TPMT *variant/*variant (homozygous deleted) n (%)	34 (92%)	3 (8%)	37