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Mercaptopurine Pharmacogenetics: Monogenic Inheritance of Erythrocyte Thiopurine Methyltransferase Activity

RICHARD M. WEINSHILBOUM[1] AND SUSAN L. SLADEK

SUMMARY

Thiopurine methyltransferase (TPMT) catalyzes thiopurine *S*-methylation, an important metabolic pathway for drugs such as 6-mercaptopurine. Erythrocyte (RBC) TPMT activity was measured in blood samples from 298 randomly selected subjects. Of the subjects, 88.6% were included in a subgroup with high enzyme activity (13.50 ± 1.86 U, mean ± SD), 11.1% were included in a subgroup with intermediate activity (7.20 ± 1.08 U), and 0.3% had undetectable activity. This distribution conforms to Hardy-Weinberg predictions for the autosomal codominant inheritance of a pair of alleles for low and high TPMT activity, $TPMT^L$ and $TPMT^H$, with gene frequencies of .059 and .941, respectively. If RBC TPMT activity is inherited in an autosomal codominant fashion, then subjects homozygous for $TPMT^H$ would have high enzyme activity, subjects heterozygous for the two alleles would have intermediate activity, and subjects homozygous for $TPMT^L$ would have undetectable activity. The segregation of RBC TPMT activity among 215 first-degree relatives in 50 randomly selected families and among 35 members of two kindreds and one family selected because they included probands with undetectable RBC enzyme activity were also compatible with the autosomal codominant inheritance of RBC TPMT. For example, in eight matings between subjects with intermediate activity (presumed genotype $TPMT^L$ $TPMT^H$) and subjects with high activity (presumed genotype $TPMT^H$ $TPMT^H$), 47% (8/17) of the offspring had intermediate activity. This value is very similar to the 50% figure expected on the basis of autosomal codominant inheritance ($\chi^2_{[1]} = .059$). Further experi-

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[1] Both authors: Clinical Pharmacology Unit, Departments of Pharmacology and Internal Medicine, Mayo Foundation, Mayo Medical School, Rochester, MN 55901.

ments are required to determine whether this genetic polymorphism for an important drug metabolizing enzyme may represent one factor in individual variations in sensitivity to thiopurines.

INTRODUCTION

The thiopurines, 6-mercaptopurine, 6-thioguanine, and azathioprine, are used in the treatment of patients with neoplastic and autoimmune diseases and in the treatment of recipients of transplanted organs [1]. Leukopenia and hepatotoxicity are included among the serious side effects of these drugs [2, 3]. It has not been possible to predict individual variations either in the therapeutic response of patients to thiopurines or in the occurrence of side effects. One factor involved in differences in response to thiopurines might be individual variations in drug metabolism. There are many examples of significant variations in drug toxicity that are due to individual differences in drug metabolism [4, 5].

The two major pathways for the metabolism of thiopurines are oxidation catalyzed by xanthine oxidase (XO) and *S*-methylation catalyzed by TPMT (fig. 1) [6, 7]. *S*-methylated thiopurines are "active metabolites" that are capable of inhibiting purine biosynthesis [8], and these compounds have been used experimentally in the treatment of leukemia [9]. Although TPMT activity in rodent liver and kidney has been studied extensively [10], it has not been well characterized in human tissue. Until recently there was no assay for the measurement of TPMT activity in an easily obtained human

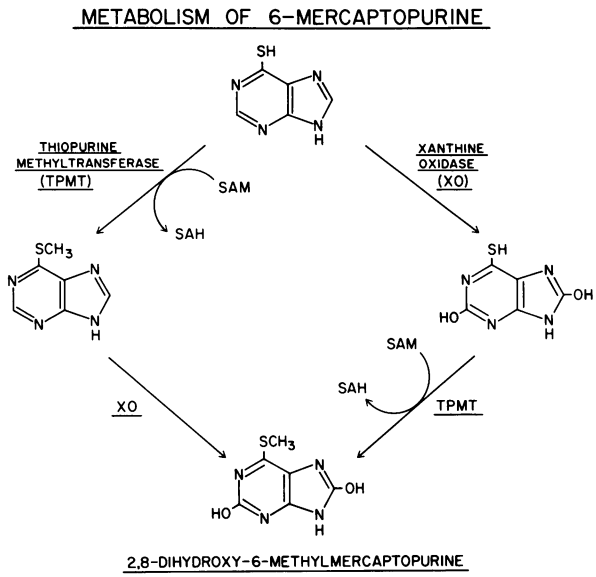

FIG. 1.—Schematic representation of 6-mercaptopurine metabolism [6, 7]. SAH = *S*-adenosyl-L-homocysteine.

tissue. However, a radiochemical assay for the measurement of TPMT activity in lysates of human erythrocytes has recently been described [11], and we have used this assay to study the role of inheritance in the regulation of human RBC TPMT activity. The results of these studies are compatible with the conclusion that the level of human RBC TPMT activity is inherited and is due mainly to a pair of alleles at a single locus. Approximately 90% of a randomly selected population is homozygous for an allele for high RBC TPMT activity, about 10% is heterozygous and has intermediate activity, and about one of every 300 subjects is homozygous for an allele for low RBC TPMT and lacks detectable enzyme activity. These observations raise the possibility that inherited variations in *S*-methylation might represent one factor involved in individual differences in the clinical response or in the occurrence of serious side effects to thiopurines.

MATERIALS AND METHODS

Subjects Studied

Blood samples anticoagulated with heparin were obtained from 298 randomly selected adult blood donors at the Mayo Clinic in Rochester, Minnesota. All subjects were white, and none was taking any medication. The average age of the randomly selected subjects was 33.9 ± 0.6 years (mean ± SEM). Blood samples were also obtained from 215 first-degree relatives in 50 families selected randomly from a list of families with children in the Rochester school system. Of the 152 families invited to participate in the study, 90 families declined to participate and 12 others were not suitable for inclusion because of divorce, the death of one parent, or the fact that the children were adopted. The average age of the 100 parents in the 50 participating families was 40.1 ± 0.5 (mean ± SEM), and the average age of the 115 children in the families was 13.0 ± 0.4. One family and two kindreds were selected for study on the basis of a proband in whom RBC TPMT activity was not detectable. The subjects in these families were informed that they had been selected because they had a relative who lacked the enzyme activity. Written consent was obtained from all subjects over 18 years, and parents gave written consent for the participation of minors.

TPMT Assay

RBC lysate TPMT activity was measured by the method of Weinshilboum et al. [11]. The assay is based on the conversion of 6-mercaptopurine to radioactively labeled 6-methylmercaptopurine with [^{14}C]*S*-adenosyl-L-methionine (SAM) as a radioactive methyl donor. Blank samples include all reagents except 6-mercaptopurine. A heated enzyme blank is not appropriate for use with this assay [11]. After incubation at 37°C for 1 hr, the reaction is stopped by the addition of borate buffer, the radioactive product is removed by organic solvent extraction, and its radioactivity is measured in a liquid scintillation counter. One U of TPMT activity is 1 nmol 6-methylmercaptopurine formed per hr/ml of packed erythrocytes.

Thiol Methyltransferase Assay

RBC membrane thiol methyltransferase (E.C.2.1.1.9) was measured by the method of Weinshilboum et al. [12]. The assay is based on the conversion of 2-mercaptoethanol to radioactively labeled *S*-methylmercaptoethanol with [^{14}C]SAM as a radioactive methyl donor. Both heated enzyme samples and samples that lack 2-mercaptoethanol are required as blanks for this reaction [12]. After incubation at 37°C for 30 min, the reaction is stopped with 1 N HCl, the radioactive product is removed by organic solvent extraction, and its radioactivity is measured in a liquid scintillation counter. One U of thiol methyltransferase activity is 1 nmol *S*-methylmercaptoethanol formed per hr/mg of membrane protein.

RBC Membrane Protein Determinations

The biuret method was used to measure protein concentrations in RBC membranes [13]. The membrane preparations were treated with 1% Triton X-100 to eliminate turbidity [14]. Bovine serum albumin was used as a standard for the protein assay.

Purification of Human Kidney TPMT

Human kidney TPMT was partially purified as described [11]. The specific activity of the partially purified enzyme was 6.9 nmol 6-methylmercaptopurine formed per hr/mg protein. This represented a 15-fold purification over the specific activity of the enzyme in the supernatant of a human kidney homogenate after centrifugation at 100,000 *g* for 1 hr.

RESULTS

RBC TPMT Activity in a Randomly Selected Population Sample

As a first step in the determination of whether there might be inherited variations in RBC TPMT, the enzyme activity was measured in blood samples from a large, randomly selected group of subjects to determine whether the population might include separate subgroups with regard to the enzyme activity level. The frequency distribution of RBC TPMT activity for these 298 randomly selected adult subjects is shown in figure 2. There was no difference between the frequency distribution histograms for male and female subjects. Approximately 90% of the subjects had relatively high TPMT activity, about 10% were included in a subgroup with intermediate activity, and

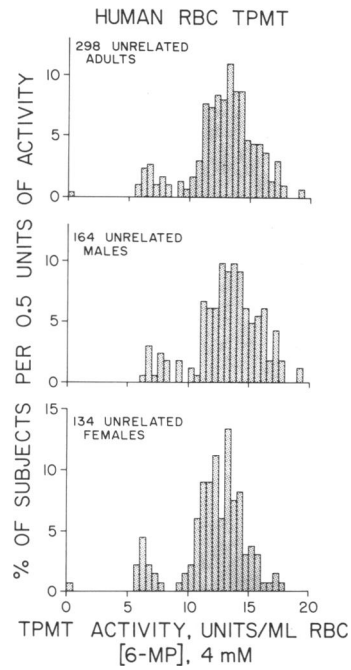

FIG. 2.—Frequency distribution of RBC TPMT activity in a randomly selected population. *Top*, RBC TPMT activity in 298 randomly selected adult blood donors; *middle*, RBC TPMT activity in 164 randomly selected male blood donors; *bottom*, RBC TPMT activity in 134 randomly selected female blood donors.

one subject had virtually undetectable enzyme activity. If the separation between high and intermediate subgroups was made at 9.5 U, the nadir of the frequency distribution, 88.6% (264/298) of the subjects were included in the high activity subgroup (> 9.5 U), 11.1% (33/298) were in the intermediate activity subgroup (1 to 9.5 U), and 0.3% (1/298) had undetectable RBC TPMT activity. The average enzyme activity for the high subgroup was 13.50 ± 1.86 U (mean ± SD, no. = 264), and for the intermediate subgroup, 7.20 ± 1.08 U (no. = 33). The average value for the entire group of 298 subjects was 12.76 ± 2.76. The assignment to subgroups was made with the knowledge that some misclassification would occur because of overlap of the distributions for the postulated high and intermediate subgroups. This distribution conformed to Hardy-Weinberg predictions for a population breeding randomly with respect to a pair of alleles for high and low enzyme activity, $TPMT^H$ and $TPMT^L$, respectively, with a gene frequency of .941 ($\sqrt{.886}$), for $TPMT^H$ and of .059 (1 − .941), for $TPMT^L$ [15].

If RBC TPMT activity is inherited in an autosomal codominant fashion, then subjects homozygous for the allele $TPMT^H$ would have high enzyme activity, subjects heterozygous for the two alleles would have intermediate activity, and subjects homozygous for the allele $TPMT^L$ would have undetectable TPMT activity. Assuming this genetic model, the gene frequency of the hypothetical allele $TPMT^H$ could be calculated on the basis of the percentage of subjects included in the high activity subgroup, and it could be determined whether the distribution of subjects between the two remaining subgroups conformed to Hardy-Weinberg predictions. The predicted distribution of 33.09 subjects in the intermediate subgroup (2 × 0.59 × .941 × 298) and 1.04 subjects in the undetectable subgroup (.059 × .059 × 298) and the actual distribution (33 intermediate and one undetectable) were identical. To determine whether other subjects with undetectable RBC TPMT activity could be identified, an additional 373 samples from blood donors were screened with a qualitative assay designed to detect only the absence of TPMT activity. One additional subject with undetectable activity was identified. Therefore, the final incidence of undetectable activity in the population sample studied was 2/671 or 0.3%. The fact that a frequency distribution of enzyme activities conforms to Hardy-Weinberg predictions does not constitute definitive proof that the enzyme activity is inherited in a monogenic (mendelian) fashion. To test the hypothesis of autosomal codominant inheritance further, RBC TPMT activity was also measured in blood samples from first-degree relatives in 50 randomly selected families.

RBC TPMT Activity in Randomly Selected Families

The frequency distribution of TPMT activity in blood samples from 215 first-degree relatives in 50 randomly selected families is shown in figure 3. The frequency distribution histogram was similar to that found for the randomly selected blood donors. Because of the inclusion of multiple offspring of these 50 families, the data for all the subjects (top panel) were biased, but the results for the 100 parents (bottom panel) were also similar to those found for randomly selected samples: 89% of the parents had values above 9.5 U. The average activity for all members of the families with activity above 9.5 U was 13.02 ± 1.47 (mean ± SD, no. = 194). The average

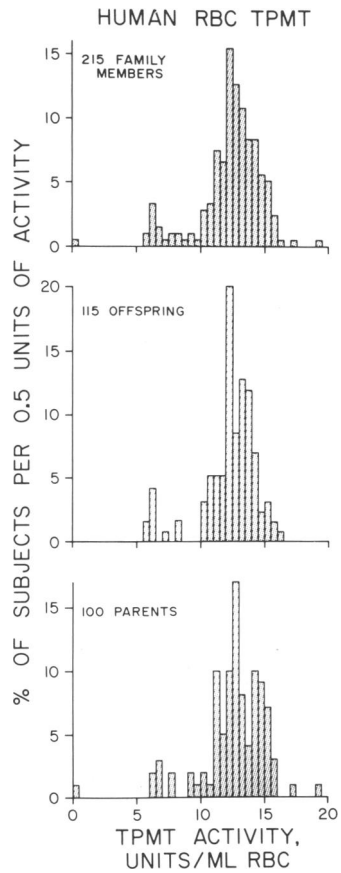

FIG. 3.—Frequency distribution of RBC TPMT activity in first-degree relatives in 50 randomly selected families. *Top*, RBC TPMT activity in 215 parents and offspring; *middle*, RBC TPMT activity in 115 offspring; *bottom*, RBC TPMT activity in 100 parents.

activity was 7.10 ± 1.11 (no. = 20) for those family members with values less than 9.5 U, excluding the one subject with undetectable activity. These results were similar to those in the 298 randomly selected subjects. Average values for parents and offspring were also very similar: 12.56 ± 2.65 (no. = 100) and 12.26 ± 2.15 (no. = 115), respectively. In addition, there was not a significant correlation of RBC enzyme activity with age for these 215 subjects (r = .061). The mating frequencies for the 50 families were very similar to the frequencies expected on the basis of random mating of subjects with respect to RBC TPMT activity: 80% (40/50) of the matings were of two individuals with high activity (> 9.5 U), 16% (8/50) included one parent with intermediate and one parent with high activity, one mating (2%) was between two parents with intermediate activity, and one mating (2%) included one parent with high and one parent with undetectable activity. This latter mating is discussed in detail below. The expected frequencies of matings of these types based on the population frequencies of RBC TPMT levels would be 78.5%, 19.7%, 1.2%, and 0.6%,

respectively. Of greater interest was the segregation within families of the TPMT activity level. In the 40 families in which both parents had high activity, none of the offspring had intermediate or undetectable TPMT activity. However, matings of one parent with intermediate activity (presumed genotype $TPMT^H$ $TPMT^L$) and one parent with high activity (presumed genotype $TPMT^H$ $TPMT^H$) yielded eight of 17 offspring with intermediate activity (fig. 4). This result was compatible with the expectation of 50% heterozygotes among matings of subjects heterozygous for a trait with subjects homozygous for high activity if autosomal codominant inheritance were assumed [16] ($\chi^2_{[1]} = .059$). In one case two subjects with intermediate activity mated, and all of the offspring had high activity (fig. 4). As will be seen below when another mating of this type is described, high-activity offspring do not always result from such matings. Overall, the data from these randomly selected families were also compatible with the hypothesis that human RBC TPMT activity is inherited in an autosomal codominant fashion. The hypothesis was strengthened further by the results of studies of kindreds selected because they included a proband with undetectable activity, an individual presumed on the basis of the hypothesis to be homozygous for the trait of low RBC TPMT activity.

Kindreds Selected on the Basis of a Proband with Undetectable RBC TPMT Activity

Pedigrees of one family and two kindreds selected because they included a proband with undetectable RBC TPMT activity are shown in figure 5. One of the probands was discovered among the initial 298 blood donors studied (family FA 37), one was included among the second group of 373 blood donors screened (family FA 35), and one was discovered incidentally during the study of randomly selected families (family FA 45). The segregation of TPMT activity in these kindreds was also compatible with

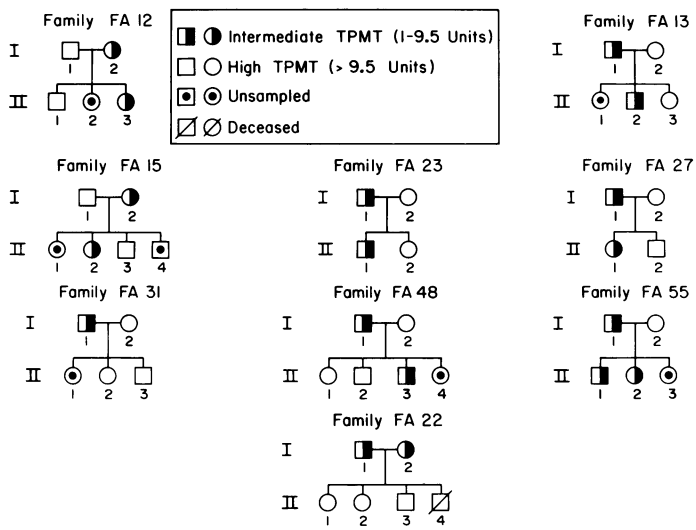

FIG. 4.—Pedigrees of nine randomly selected families in which at least one parent had intermediate TPMT activity.

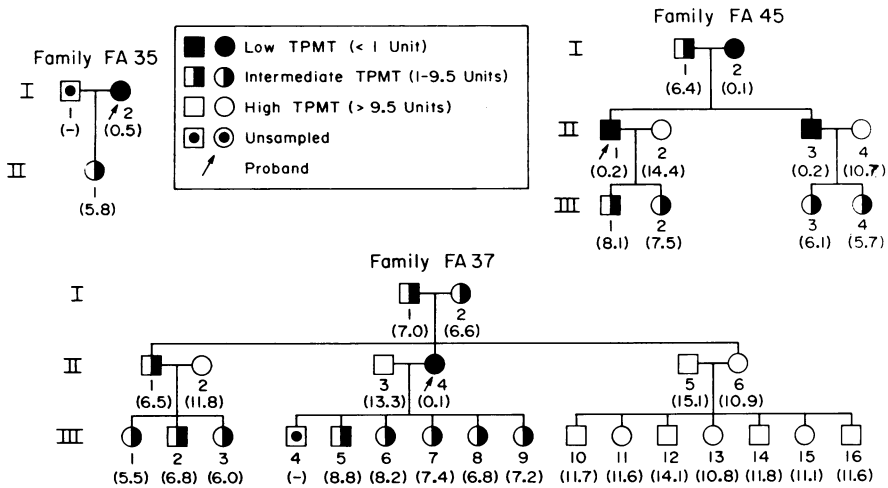

FIG. 5. —Pedigrees of two kindreds and one family that included a proband with undetectable RBC TPMT activity (< 1.0 U). RBC TPMT activity values for each subject shown in *parentheses*.

the hypothesis of autosomal codominant inheritance of RBC TPMT activity. All of the nine offspring studied who resulted from matings between a parent with undetectable activity and a parent with high activity had intermediate RBC TPMT, even though subjects with intermediate activity made up only 11.1% of a randomly selected population. Two sets of parents of subjects with undetectable activity were available for study. In one case each of the parents had intermediate activity, and in the other case one parent had intermediate and one had undetectable activity. The assignment of members of these kindreds to undetectable, intermediate, or high TPMT activity subgroups was relatively unambiguous (see enzyme activity levels in fig. 5). Although TPMT values for subjects with "undetectable" RBC enzyme values are shown in figure 5, these values were so low that they had no real meaning. Once again, the results were compatible with the hypothesis that human RBC TPMT activity is inherited as an autosomal codominant trait, and that subjects homozygous for the allele for low activity have virtually undetectable RBC TPMT activity.

"Mixing" Experiments and Experiments with the Addition of Purified TPMT

If variations in RBC TPMT activity are genetically determined, they might be due to inherited differences in endogenous enzyme activators, inhibitors, or competing enzyme systems rather than to variations in TPMT activity itself. To test this possibility, experiments were performed in which lysates from five subjects with undetectable RBC TPMT activity were mixed with a pooled lysate from four subjects with high RBC enzyme activity. Both equal volume mixtures and mixtures of 3 parts of either low or high activity lysate with 1 part of the lysate of the alternative level of enzyme activity were made. The final TPMT activities in these mixtures were the expected additive values (table 1). In addition, partially purified human kidney TPMT was added to lysates from four subjects with high activity and to lysates from the five subjects with undetectable activity. The recovery of added partially purified enzyme

TABLE 1

HUMAN RBC TPMT: MIXING EXPERIMENTS

Mixture	cpm observed	cpm expected
Low, 100%	10 ± 8	...
Low:High, 3:1	317 ± 10	301 ± 6
Low:High, 1:1	613 ± 12	592 ± 6
Low:High, 1:3	907 ± 10	882 ± 5
High, 100%	1173 ± 5	...

NOTE. —TPMT activity expressed as counts per minute (cpm) in mixtures of various quantities of RBC lysate from five individual subjects with undetectable enzyme activity with a pooled sample of lysate from four subjects with high enzyme activity. Values are mean ± SEM for three determinations.

activity from these two types of samples was virtually identical (table 2). The results of these two series of experiments make it unlikely that the inherited variations in TPMT activity found in this study result from inherited differences in TPMT activators, inhibitors, or competing enzyme systems.

Lack of Correlation with Erythrocyte Thiol Methyltransferase Activity

In spite of the important role played by TPMT in thiopurine metabolism, confusion has existed regarding its nomenclature. Some authors have referred to this activity as "thiol methyltransferase" (E.C.2.1.1.9) [17], although that name has usually been reserved for a membrane-associated enzyme that catalyzes the *S*-methylation of aliphatic sulfhydryl compounds such as 2-mercaptoethanol [18]. The initial descrip-

TABLE 2

HUMAN RBC TPMT

	RECOVERY OF TPMT	
SAMPLE	U	%
Undetectable activity group:		
FA 35, I-2	22.2	101
FA 37, II-4	22.1	101
FA 45, I-2	22.0	100
FA 45, II-1	22.1	101
FA 45, II-3	21.9	99
Mean ± SEM	22.1 ± 0.1	100.4 ± 0.4
High activity group:		
FA 32, I-2	22.2	101
FA 38, I-2	22.4	102
FA 45, II-2	21.1	96
FA 47, I-1	21.4	98
Mean ± SEM	21.8 ± 0.3	99.3 ± 1.4

NOTE. —Addition of partially purified human kidney TPMT to RBC lysates of five subjects with undetectable RBC TPMT and lysates of four subjects with high RBC TPMT. Average activity for the four subjects with high activity was 11.97 ± 0.16 U (mean ± SEM). Each value represents the mean of three determinations.

tions of TPMT clearly differentiated it from the membrane-bound thiol methyltransferase activity [10]. Although RBC lysates do not contain thiol methyltransferase activity [12], human RBC membranes do have such an activity [12]. Therefore, thiol methyltransferase activity was measured in RBC membranes from 131 samples obtained from the blood of the family members in whom RBC lysate TPMT had also been measured. There was not a significant correlation of these two *S*-methylating activities (r = .073).

DISCUSSION

The experiments described here were designed as a first step in an investigation of the possibility of significant genetic variations in the metabolism of thiopurines. The activity of an important thiopurine metabolizing enzyme activity, TPMT, has been measured in an easily obtained tissue, the human RBC, and the results are compatible with the conclusion that the level of RBC enzyme activity is inherited as an autosomal codominant trait. Approximately 11% of randomly selected subjects have intermediate TPMT activity, and one of every 300 subjects has undetectable RBC enzyme activity on the basis of this genetic polymorphism. Whether the subjects with very low activity actually lack RBC enzyme activity, and whether enzymatically inactive TPMT protein is present in their RBCs, will have to be determined in the future. We have proposed that the locus responsible for this polymorphism be referred to as *TPMT*, while the alleles for low and high enzyme activity be designated $TPMT^{L}$ and $TPMT^{H}$, respectively. These designations conform to the recommendations of the Committee on Nomenclature of the Third International Workshop on Human Gene Mapping [19]. Our observations do not rule out the possibility that other factors, either genetic or environmental, will also be found to regulate human TPMT activity.

No conclusion can yet be drawn as to whether this genetic polymorphism in TPMT activity may represent an important factor in individual sensitivity to thiopurines or to the thiopyrimidine drugs such as propylthiouracil that may also be methylated by TPMT [10, 17]. It must first be determined whether the relative levels of RBC enzyme activity reflect the relative levels of TPMT activity in other tissues, and, if so, whether the inherited variation is of functional significance in the metabolism of thiopurines. Inherited variations in the *N*-acetyltransferase activity that plays such an important role in the occurrence of side effects for the drugs hydralazine and isoniazid [4, 5] cannot be measured in human blood [20]. However, the level of RBC catechol-*O*-methyltransferase activity, an important catecholamine and catechol drug-metabolizing enzyme, is under genetic regulation [21–23], RBC catechol-*O*-methyltransferase activity reflects the level of enzyme activity in other tissues [24], and variations in RBC catechol-*O*-methyltransferase activity are correlated with individual variations in the *O*-methylation of catechol drugs such as L-dopa [25]. Whether the situation with the genetic polymorphism for RBC TPMT activity will prove to be analogous to that for RBC catechol-*O*-methyltransferase remains to be determined. The observations reported here raise the possibility that inherited variation in TPMT activity may represent one factor in individual variations in sensitivity to thiopurine drugs and in the occurrence of serious side effects when these drugs are used clinically.

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