DRUG BIOCHEMISTRY

THIOPURINE S-METHYLTRANSFERASE PHENOTYPE-GENOTYPE CORRELATION IN CHILDREN WITH ACUTE LYMPHOBLASTIC LEUKEMIA

MARIA CHRZANOWSKA[1], MARTA KUEHN[1], DANUTA JANUSZKIEWICZ-LEWANDOWSKA[2], MATEUSZ KURZAWSKI[3] and MAREK DROŹDZIK[3]*

[1]Department of Physical Pharmacy and Pharmacokinetics, University of Medical Sciences, Święcickiego 6, 60-781 Poznań, Poland
[2]Department of Pediatric Oncology, Hematology and Transplantology, University of Medical Sciences, Szpitalna 27/33, 60-572 Poznań, Poland
[3]Department of Pharmacology, Pomeranian Medical University, Powstańców Wlkp. 72, 70-111 Szczecin, Poland

Abstract: Thiopurine S-methyltransferase (TPMT) is an enzyme that catalyzes the S-methylation of thiopurine drugs such as 6-mercaptopurine, 6-thioguanine, and azathioprine. TPMT activity exhibits an interindividual variability mainly as a result of genetic polymorphism. Patients with intermediate or deficient TPMT activity are at risk for toxicity after receiving standard doses of thiopurine drugs. The aim of this study was to determine the *TPMT* genotype and phenotype (activity) and investigate the correlation between *TPMT* genotype and enzyme activity in 43 Polish children receiving 6-MP during maintenance therapy in course of acute lymphoblastic leukemia (ALL), in 16 children with ALL at diagnosis and 39 healthy controls. TPMT activity was measured in RBC by HPLC method. Patients were genotyped for *TPMT *2, *3A* and *3C* variant alleles using PCR-RFLP and allele-specific PCR methods. In the group of children with ALL during maintenance therapy, median TPMT activity (29.3 nmol 6-mMP g^{-1} Hb h^{-1}) was significantly higher compared to the group of children with ALL at diagnosis (20.6 nmol 6-mMP g^{-1} Hb h^{-1}, p = 0.0028), as well as to the control group (22.8 nmol 6-mMP g^{-1} Hb h^{-1}, p = 0.0002). Percentages of individuals heterozygous for *TPMT* variant allele in respective groups were: 9.3, 6.2 and 15.5% (p > 0.05). In all the study groups heterozygous patients manifested a significantly lower TPMT activity as compared to the wild type homozygotes (16.7 ± 2.1 *vs.* 31.2 ± 6.8 nmol 6-mMP g^{-1} Hb h^{-1}, p = 0.002, in children during maintenance therapy, 11.9 ± 2.7 *vs.* 24.6 ± 9.5, p = 0.0003, in the combined group of children with ALL at diagnosis and controls). The results present that commencement of the thiopurine therapy caused an increase in the TPMT activity in RBCs by approximately 20%. All patients heterozygous for the *TPMT* variant allele revealed decreased TPMT activity compared to *TPMT* wild-type patients. Since decreased TPMT activity is associated with higher risk for toxicity after receiving standard doses of thiopurine drugs, pretreatment determination of TPMT status, with phenotypic or genetic assay, should be performed routinely, also in Poland.

Keywords: thiopurine S-methyltransferase, TPMT, pharmacogenetics, acute lymphoblastic leukemia

Thiopurine S-methyltransferase (TPMT) is a cytosolic enzyme that catalyzes the S-methylation of aromatic and heterocyclic sulfhydryl compounds, including the thiopurine drugs such as 6-mercaptopurine (6-MP), azathioprine (AZA) and 6-thioguanine (6-TG). These drugs are widely used in the treatment of patients suffering from various diseases: as antimetabolites in cancer, immunosuppressants in autoimmune disorders, and in prevention of acute rejection after organ transplantation.

6-MP, which is a purine antymetabolite, has been used for many years along with other drugs in treatment of leukemia in children (1–3). It is a substantial drug in the remission maintenance chemotherapy in children with acute lymphoblastic leukemia (ALL). Studies carried out over many years have shown that, despite using the same 6-MP doses, there are significant interpersonal differences in the concentrations of 6-MP active metabolites in red blood cells (RBC). Those differences are associated with the relapse of the disease. In recent years, it has been demonstrated that the differences in the therapeutic efficacy and adverse effects of 6-MP in individual subjects are associated with the genetic polymorphism of the TPMT (4, 5), which is the key enzyme in 6-MP metabolism. 6-MP is a prodrug and its metabolism involves three competing routes. The two catabolic ones take places in the liver and are

* Corresponding author: e-mail: drozdzik@pum.edu.pl

the effect of xanthine oxidase (XO) and TPMT. An anabolic route takes places intracellularly and is catalyzed by hypoxanthine guanine phosphoribosyltransferase, it leads to form 6-thioguanine nucleotides. 6-TGNs can be incorporated into DNA, and their levels are highly correlated with thiopurine toxicity and therapeutic efficacy.

The distribution of the TPMT activity in Caucasian population is trimodal. Approximately 90% of subjects exhibit high TPMT activity (TPMT^H/TPMT^H homozygotes), 10% have intermediate activity (TPMT^H/TPMT^L heterozygotes), whereas 0.3% of subjects demonstrate low or undetectable activity of this enzyme (TPMT^L/TPMT^L homozygotes) (6). TPMT polymorphism is a model example of use of pharmacogenetics for optimization of the therapy. There are several mutant alleles associated with lower TPMT activity. Based on studies in various world populations to date, *TPMT*3C*, **3A** and **2** are predominant variant alleles, over 95% of inherited TPMT deficiency. Other mutations are rare or were found only in a single individuals (7, 8). TPMT activity is inversely related to erythrocyte 6-thioguanine nucleotides (6-TGN) concentration. Individuals with TPMT deficiency reach very high concentrations of the cytotoxic 6-TGNs, which predisposes to develop life-threatening myelosuppression if standard doses of the thiopurine drugs are used (9). TPMT deficient patients should be treated with 5 to 10% of the standard dose of the thiopurine drugs to avoid toxicity (10, 11). Intermediate TPMT activity also predisposes to myelotoxicity. It has been demonstrated that, despite medium TPMT activity, symptoms of myelosuppresion occurred in some patients without mutations (homozygotes) (12). This is due to the fact that some homozygotes exhibit slightly lower TPMT activity than the conventionally accepted separating the wild type homozygotes from heterozygotes. Due to 30-fold overall and 3- to 4-fold variability in TPMT activity between heterozygous and wild type homozygous patients (7, 13) phenotyping is an equally important aspect of the clinical diagnosis. Such variability cannot be detected only by genotyping procedures. The therapeutic use of TPMT testing relates to its ability to identify those patients with enzyme deficiency (0.3–0.6%) who will almost certainly develop myelosuppression after standard doses of the thiopurine drugs. It is also considered that attainment of adequate 6-TGN concentrations and their maintenance during entire remission maintenance therapy is an important factor determining the success of treatment, regardless of other prognostic factors. In our opinion, all individuals commencing thiopurine therapy should undergo prospective screening (genotype and/or phenotype) for TPMT status.

The aim of this study was to determine the *TPMT* genotype and phenotype (activity) and investigate the correlation between *TPMT* genotype and enzyme activity in Polish children receiving 6-MP during maintenance therapy and in children with ALL at diagnosis.

EXPERIMENTAL

Patients

Peripheral blood samples were collected from 43 children with ALL during first remission maintenance therapy, aged between 1 and 17 years (mean 6.3 ± 4.8). The group consisted of 21 boys (48.8%, mean age 7.1 ± 4.5) and 22 girls (mean age 6.6 ± 4.4). The study also included 16 children with ALL with diagnosis. Mean age in this group of patients was 6.6 ± 3.5 (aged from 2 to 16) and this group consisted of 8 boys (50.0%, mean age 6.1 ± 2.4) and 8 girls (mean age 7.0 ± 4.5). The control group comprised 39 healthy children aged between 2 and 15 years (mean age 7.3 ± 3.9). This group included 18 boys (46.1%) aged between 2 and 15 years (mean age 7.3 ± 3.9) and 21 girls aged 2 to 14 years (mean age 6.7 ± 4.1). In all groups both genotype and phenotype were determined. The children with the first remission of ALL were receiving 50 mg/m^2 of 6-MP a day and 20 mg/m^2 of methotrexate (MTX) once a week. The study was conducted in compliance with the recommendations of the Declaration of Helsinki with respect to human subjects and was approved by the Bioethical Commission at the University of Medical Sciences in Poznań.

Phenotyping

TPMT activity HPLC assay was performed according to Kröplin (14, 15).

Genotyping

Genomic DNA was isolated from white blood cells obtained from 450 μL of whole blood samples using the detergent, non-enzymatic extraction method. Three common *TPMT* variants influencing enzyme activity: G238C (*TPMT*2*), A719G (*TPMT*3C*), and G460A + A719G (*TPMT*3C*), were determined in each patient using previously described PCR-based assays (16).

Statistical analysis

TPMT activity values were compared using non-parametric statistics test (Mann-Whitney's
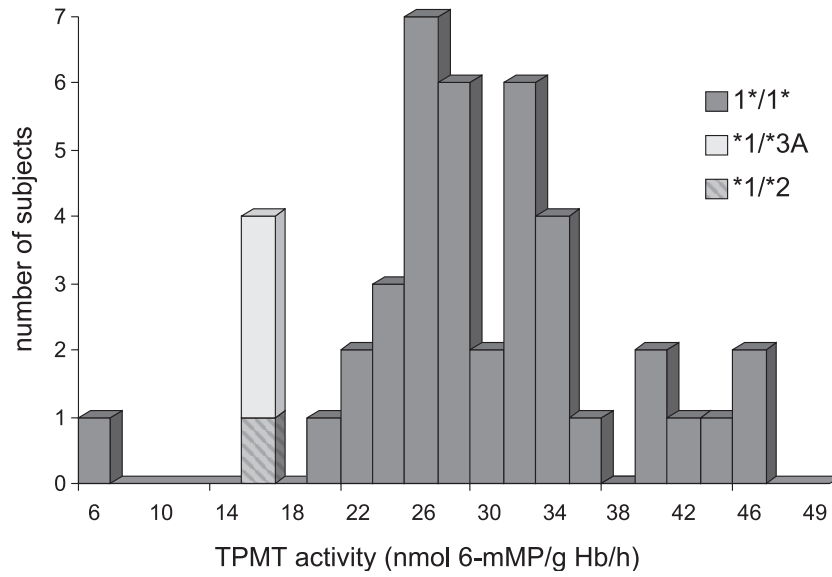
Figure 1. Distribution of *TPMT* activity in comparison with determined *TPMT* genotype in children with ALL during maintenance therapy
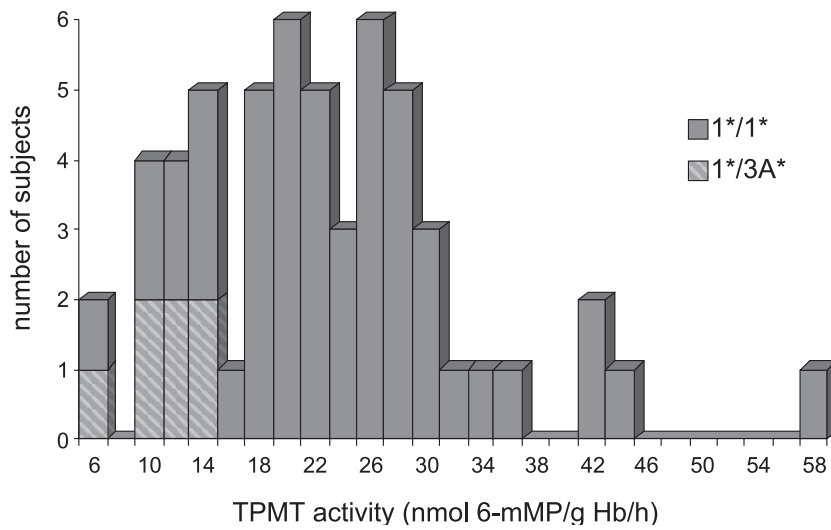

Figure 2. Distribution of TPMT activity in comparison with determined *TPMT* genotype in children with ALL at diagnosis and in the control group

Table 1. Activity of TPMT in RBCs of examined children.

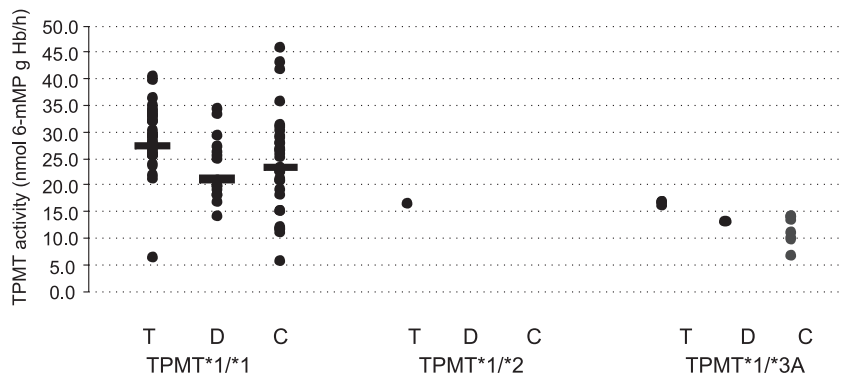| Group	TPMT activity (nmpl 6-mMP/g Hb/h) Median value (range)			Mann-Whitney test
	Total	Girls	Boys	
Children during therapy (T) n = 43	29.3 (6.6–47.1)	27.3 (n = 22) (16.3–46.8)	30.0 (n = 21) (6.6–47.1)	NS
Children at diagnosis (D) n = 16	20.6 (13.4–33.5)	26.6 (n.=.8) (13.4–33.5)	19.7 (n.=.8) (14.0–26.3)	NS
Control group (C) N = 39	22.8 (6.0–45.8)	23.3 (n.=.18) (6.0–45.8)	22,8 (n.=.21) (6.9–43.6)	NS

Figure 3. Correlation of *TPMT* genotype and phenotype in children with ALL during maintenance therapy (T), children with ALL at diagnosis (D) and control group (C). Horizontal lines represents the median activities

test); p values of less than 0.05 were considered to be statistically significant. Confidence interval (CI 95%) method was used to compare the frequencies of TPMT alleles in examined groups. The correlations between TPMT activity with respect to age and gender in each group were analyzed with Student *t*-test.

RESULTS

In the group of 43 children with ALL during maintenance therapy activity ranged between 6.6 to 47.1 nmol 6-mMP g^{-1} Hb h^{-1} (median value of 29.3). Median TPMT activity was 30.0 nmol 6-mMP g^{-1} Hb h^{-1} and 27.3 nmol 6-mMP g^{-1} Hb h^{-1} for boys (n = 21) and girls (n = 22), respectively. The TPMT activity in the group of 16 children with ALL at diagnosis ranged from 13.4 to 57.0 nmol 6-mMP g^{-1} Hb h^{-1} (median value of 20.6). Median TPMT activity was 19.7 nmol 6-mMP g^{-1} Hb h^{-1} and 26.6 nmol 6-mMP g^{-1} Hb h^{-1} for boys (n = 8) and girls (n = 8), respectively. In the control group (n = 39) TPMT activity ranged from 6.0 to 45.8 nmol 6-mMP Hb h^{-1} and median TPMT activity was 22.8 nmol 6-mMP g^{-1} Hb h^{-1}. Median TPMT activity was 22.8 nmol 6-mMP g^{-1} Hb h^{-1} for boys (n = 18), whereas in girls (n = 21) were 23.3 nmol 6-mMP g^{-1} Hb h^{-1}, respectively. There was no statistically significant differences in TPMT activity in relation to gender and age in any of the group (Tab. 1). Median TPMT activity in the group of children with ALL during maintenance therapy (29.3 nmol 6-mMP g^{-1} Hb h^{-1}) was higher than in children with ALL at diagnosis (20.6 nmol 6-mMP g^{-1} Hb h^{-1}) and in a control group (22.8 nmol 6-mMP g^{-1} Hb h^{-1}). The differences were

statistically significant (p = 0.0028 and p = 0.0002, respectively). No statistically significant differences were observed between TPMT activity in children at the time of ALL diagnosis and TPMT activity in the control group (p > 0.05).

Among 43 children with ALL during maintenance therapy, 39 (90.7%) were wild type *TPMT*1/*1* homozygous and 4 were heterozygous (9.3%). Three of the heterozygous patients were classified as a *TPMT*1/*3A* (7.0%) and one as a *TMPT*1/*2* (2.6%). The group of 16 children with ALL at diagnosis contained 15 wild type *TPMT*1/*1* homozygotes (93.8%) and 1 *TPMT*1/*3A* heterozygote. In the control group, 33 children (84.6%) were wild-type homozygous and 6 children (15.4%) were heterozygous for the *TPMT*3A* variant allele. In all of the groups the heterozygous patients manifested a significantly lower TPMT activity as compared to the wild type homozygotes (31.2 ± 6.8, as compared to 16.7 ± 2.1 nmol 6-mMP g^{-1} Hb h^{-1} in children during maintenance therapy and 24.6 ± 9.5 as compared to 11.9 ± 2.7 nmol 6-mMP g^{-1} Hb h^{-1} in both groups of children with ALL at diagnosis and control).

Each group of children participating in the study included subjects who exhibited decreased TPMT activity, but were not heterozygotes (Fig. 1 and 2). Figure 3 represents the distribution of TPMT activity in comparison with determined TPMT genotype in children from all groups.

In a total of 98 examined children we detected 88.8% (n = 87) carrying wild type *TPMT*1/*1* genotype and 11.2% (n = 11) of heterozygotes, including 10.2% *TPMT*1/*3A* (n = 10) and 1.0% *TPMT*1/*2* (n = 1). The wild type *TPMT*1* accounted for 94.4% (185/196) alleles. *TPMT*3A*

accounted for 5.1% (10/196) alleles and *TPMT*2* 0.5% (1/196) alleles.

DISCUSSION AND CONCLUSION

It is now well known that genetic polymorphisms that modify the enzymatic activity of TPMT can contribute to interindividual differences in susceptibility to thiopurine therapy. TPMT plays an important role in the metabolism of the thiopurine drugs and affects not only the efficacy but also the safety of treatment. Individuals who inherited one or two defective *TPMT* alleles can develop severe hematopoietic toxicity when treated with standard doses of 6-MP because of the accumulation of cytotoxic metabolites. Clinical practice in Poland does not include routine TPMT genotyping and/or phenotyping in children with ALL, even though the enzyme is of key importance for 6-MP metabolism.

In the above presented study, TPMT genotype and phenotype have been evaluated in Polish children with diagnosed ALL and in children with ALL receiving 6-MP in remission maintenance therapy. The results of our study have shown that the TPMT activity in children with ALL receiving 6-MP during remission maintenance therapy was 20% higher than in children at the time of ALL diagnosis. No significant differences in TPMT activity with respect to gender and age have been observed. TPMT activity in children at the time of diagnosis did not differ as compared to healthy subjects from the control group. These results can be compared with other studies, which indicate that commencement of the thiopurine therapy causes an increase in the TPMT activity in RBCs by approximately 20%, especially in heterozygous patients (17).

It is now recognized that TPMT genotyping or phenotyping should be performed prior to administration of the thiopurine drugs in order to predict the risk of developing severe hematotoxicity in patients. However, both genotyping and phenotyping procedures have their limitations. The measurement of TPMT activity in RBC can be sometimes misleading, for example in patients who had received a recent blood transfusion. It is also well known that several currently used drugs, such as salicylic acid derivatives (mainly sulfasalazine), trimethoprim and diuretics can alter TPMT activity (18–22). On the other hand, genotyping assays have been proposed to overcome these limitations. Determination of three variant alleles most common in Caucasian population (*TPMT*2, TPMT*3A and TPMT*3C*) allows to identify 90 to 95% of patients with altered activity of the enzyme. However, routine *TPMT* genotyping assays do not include rare variants, potentially influencing enzyme activity. It was estimated on base of results in a German population that rare coding variants may be present in about 0.3% of Caucasian subjects (7).

The results of our study have shown that the TPMT activity, the allele frequencies and distribution among patients were similar to those found in other Caucasian populations: with the *TPMT*3A* as a main variant allele (frequency 10.2%) as well as 11.2% frequency of heterozygous patients (7, 8, 23–25). In the present study, ten heterozygous carriers for *TPMT*3A* and one carrier for *TPMT*2* were found, and all eleven patients revealed intermediate TPMT activity, much lower than the TPMT wild-type patients (p = 0.002 for children during remission maintenance therapy and p = 0.0003 for children at the time of ALL diagnosis and control subjects). This clearly substantiates that *TPMT*3A* is associated with decreased TPMT activity even in heterozygous subjects, leading to an intermediate TPMT phenotype. The activity of TPMT correlates with *TPMT* genotype. However, in each group of children participating in the study, some intermediate TPMT activity individuals were found, not carrying any of the analyzed alleles. It is convergent with the previous data, indicating that about 2 to 5% of subjects exhibit depressed activity of the enzyme and are characterized as intermediate metabolizers, despite not carrying any of the common variant of the TPMT alleles (7, 8, 25). Rare mutations in the *TPMT* gene and other genetic factors may underlay the lack of paralelism. Alternatively, enviromental factors, like chemicals applied as drugs or diet components, might have resulted in decreased activity in individuals genotyped as *TPMT* wild-type. The results demonstrate discordance between *TPMT* genotype and enzyme activity in some patients. One can conclude that phenotyping still remains an equally important element of clinical diagnostics and cannot be simply substituted by genotyping in all cases. This is especially important in heterozygous patients, who manifest two- or even threefold variability in TPMT activity.

In our opinion all individuals commencing thiopurine therapy should undergo prospective screening for TPMT status. Coupling both methods – genotyping and phenotyping – together would result in an increased sensitivity of identifying patients with altered TPMT activity. However, the main clinical value of TPMT diagnostics lies in the ability to identify patients completely deficient for TPMT activity, who develop profound myelosuppression after standard thiopurine doses in all cases.

In case of those patients, both methods can be used, however, lower costs and faster procedure point to genotyping as a method of choice for routine TPMT status testing before the initiation of mercaptopurine therapy. In many countries, i.e., USA, Spain or Germany TPMT testing has been already introduced into clinics. We suggest that pretreatment determination of TPMT status and pharmacogenetically guided thiopurine therapy should be performed routinely also in Poland.

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