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ORIGINAL ARTICLE

Thiopurine methyltransferase polymorphisms and mercaptopurine tolerance in Turkish children with acute lymphoblastic leukemia

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Abstract
Purpose Thiopurine methyltransferase (TPMT) enzyme is involved in the metabolism of 6-mercaptopurine (6-MP), a key component of acute lymphoblastic leukemia (ALL) treatment protocols in children. The aims of this study were to investigate the frequency of common genetic polymorphisms associated with low TPMT activity and correlations of polymorphic variants with 6-MP tolerance in a group of Turkish children with ALL.
Methods Genotyping for G238C, A719G, and G460A mutations were performed by using NanoChip Technology. Adverse reactions during the first 6 months of maintenance therapy with oral 6-MP and methotrexate were retrospectively analyzed from patient's files.
Results Five (8.6%) of 58 children with ALL had a polymorphic TPMT allele: 4 (3.4%) were heterozygous for TPMT*3A (G460A and A719G), and one (0.9%) was heterozygous for TPMT*3C (A719G). No cases with TPMT*3B (G460A) or TPMT*2 (G238C) variants were identified. Children with TPMT*3A and *3C had significantly lower leukocyte and neutrophil counts and percentage of target 6-MP dosage, and longer periods with ≥grade 2 infections, ≥grade 2 liver toxicity, and chemotherapy interruptions than the children with wild-type TPMT during the first 24 weeks of maintenance therapy.
Conclusions The frequency and distribution of common TPMT polymorphisms in Turkish children with ALL is similar to other Caucasian populations. Polymorphic variants were associated with excessive 6-MP toxicity supporting the suggestion that TPMT genotyping should be performed before institution of 6-MP therapy.

Keywords Thiopurine methyltransferase polymorphisms · Mercaptopurine · Children · Leukemia

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Introduction

Thiopurine methyltransferase (TPMT) is a cytosolic enzyme involved in the inactivation of thiopurine drugs such as 6-mercaptopurine (6-MP), thioguanine and azathiopurine [1, 2]. The 6-MP is a key component of chemotherapy protocols used in childhood acute lymphoblastic leukemia (ALL) [3]. The activity of TPMT enzyme is decreased in 6–11% of general population due to single nucleotide polymorphisms in TPMT gene and undetectable in 1/300 individuals carrying two mutant alleles [4, 5]. Low TPMT level has been associated with increased bone marrow toxicity following treatments with thiopurine drugs [6, 7]. Several TPMT polymorphic alleles associated with low enzyme activity have been described, the most common of them being TPMT*3C (A719G), TPMT*3B (G460A), TPMT*3A (G460A and A719G), and TPMT*2 (G238C), which account for 89–95% of inherited TPMT deficiency [8–10]. The frequencies of these non-functional TPMT polymorphisms vary widely among different populations [11–20]. The TPMT*3A is the most frequent allele in Caucasians while it is uncommon or absent in Eastern Asia, India, and Africa where TPMT*3C is the commonest TPMT allele.

The aims of this study were to investigate the frequency of common TPMT polymorphisms associated with low enzyme activity in a cohort of Turkish children with ALL and to examine the correlations of polymorphisms with 6-MP toxicity during maintenance therapy.

Patients and methods

Patients

All children with ALL who attended the Pediatric Hematology Clinics of Gazi University Hospital between August 2008 and December 2009 were consecutively enrolled after obtaining written informed consent from the parents, and the study was approved by the Institutional Review Board. A total of 58 patients (35 males and 23 females) were eligible for TPMT genotyping. They can serve as a representative of Turkish population since their families were originated from different provinces of the country.

All of the study patients were treated at our institution according to the ALL-BFM (Berlin Frankfurt-Münster)-95 Protocol as described elsewhere [21]. Of 58 children, 51 were eligible for toxicity analysis since they had received at least the first 24 weeks of maintenance therapy. The remaining seven children were excluded from toxicity analysis since they did not receive maintenance therapy (six patients were still on the earlier courses of chemotherapy protocol and one high-risk patient underwent stem cell transplantation after intensification chemotherapy). The maintenance therapy of ALL-BFM-95 protocol consisted of daily 6-MP, 50 mg/m^2, PO and weekly methotrexate (MTX), 20 mg/m^2, PO. The doses of both drugs were adjusted to keep white blood cell (WBC) count between 2,000/μl and 3,000/μl. They were reduced by 50% if WBC count was between 1,000/μl and 2,000/μl, increased by 150% if WBC >3,000/μl and withdrawn when WBC <1,000/μl or SGOT/SGPT level was ≥5 times the upper limit of normal range or ≥grade 2 neutropenic infection was present. The WBC count, absolute neutrophil count (ANC), and percentage of periods with leucopenia, neutropenia, chemotherapy interruptions, with and without infections, and with ≥grade 2 liver toxicity as well as 6-MP dose were recorded as a percentage of the total target dose in each patient. Infectious, hematologic, and liver toxicity data for purpose of this study were collected using National Cancer Institute (NCI) criteria.

TPMT genotype analysis

DNA extraction

Blood sample (4 ml) was collected in a tube with EDTA. Genomic DNA was extracted using NucleoSpin kit (Macherey–Nagel GmbH, Düren, Germany) according to the protocol provided by the manufacturer. Absorbance was measured at 260 and 280 nm with a ND-1000 spectrophotometer (NanoDrop, USA); the A_{260}/A_{280} ratio and the DNA concentration were calculated. The DNA concentration was adjusted to allow 100 ng to be used for PCR. The DNA samples were stored at −20°C. Genotyping for G238C, A719G, and G460A mutations were performed by using NanoChip Tecnology (Nanogen, Inc San Diego,CA). Three regions of TPMT gene were amplified by using specific oligonucleotide primers (Montwell, Turkey). PCR was carried out for each patient in a total volume of 25 μl containing 17.2 μl dH_2O, 0.5 μl of 10 mM dNTPs, 2.5 μl of 10× PCR buffer with 15 mM $MgCl_2$, 1.5 μl of 25 mM $MgCl_2$, 1 μl of 10 pmol primer, 0.3 μl of 5 U/ml *Tag* DNA polymerase, and 2 μl of 100 ng DNA. PCR was performed with GeneAmp PCR System 9700 (Applied Biosystems). The temperature profile included an additional denaturation step of 12 min at 94°C, followed by 35 cycles of a denaturation step at 94°C for 1 min, a primer annealing step at 60°C for 1 min, and an extension step at 72°C for 1 min, with final extension step at 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel. The gel contained 10 μl ethidium bromides.

The amplicons was prepared for the detection of all mutations with NanoChip Technology. After amplification, purification procedure was performed, for desalting and loading on to the cartridge. The purified products were checked by electrophoresis on a 2% agarose gel. The purified samples were loaded in different wells for each patient. The samples were analyzed by different reporter mixes for each mutation. The occurrence of the mutations has been evaluated by ratio references that include both wild-type and mutant type signals.

Statistical analysis

Statistical analysis was performed using SPSS-11.5. Data are expressed in mean ± SD. Differences between groups were analyzed using the Mann–Whitney U test and frequencies by the Fischer exact test. Correlations were calculated by Spearman test. P values <0.05 were considered statistically significant.

Results

Frequency of TPMT alleles

Of the 58 children with ALL, five (8.6%) had a mutated TPMT allele. Four children were heterozygous for both the A719G and G460A polymorphisms (TPMT*3A) and one was heterozygous for only A719G polymorphism (TPMT*3C). Allele frequencies for TPMT*3A and TPMT*3C among 58

children with ALL were 3.4 and 0.9%, respectively. No homozygosity for these polymorphisms was found. None of the three children with TPMT*3A had an ancestral relation. The G289A (TPMT*2A) polymorphism was not identified in our study group (Table 1).

Bone marrow and liver toxicity

The toxic side effects observed during the first 24 weeks of maintenance chemotherapy were summarized in Table 2. There were significant correlation between the 6-MP dose and WBC count ($r = 0.86$, $P < 0.05$) and ANC ($r = 0.68$, $P < 0.05$) in 51 children (Figs. 1, 2). One of the five children with a variant TPMT underwent stem cell transplantation, and toxicity analysis was performed in the remaining four children.

The mean values of WBC, ANC counts and percentage of 6-MP dose were significantly lower, and percentage of periods with maintenance of leukocyte count below 2,000/μl, infectious episodes ≥grade 2, liver toxicity ≥grade 2, and

Table 1 The frequency of TPMT alleles in 58 Turkish children with acute lymphoblastic leukemia

TPMT alleles	Polymorphisms	No. of alleles	Allele frequency (%)	95% CI
Total alleles		116		
TPMT*1/*1	Wild type	106	91.4	85.1–99.5
TPMT*2	G289A	0	0	–
TPMT*3A/*1	A719G/G460A	4	3.4	0.7–6.2
TPMT*3B	G460A	0	0	–
TPMT*3C/*1	A719G	1	0.9	0.1–1.98
Total mutant alleles		5	4.3	1.9–6.4

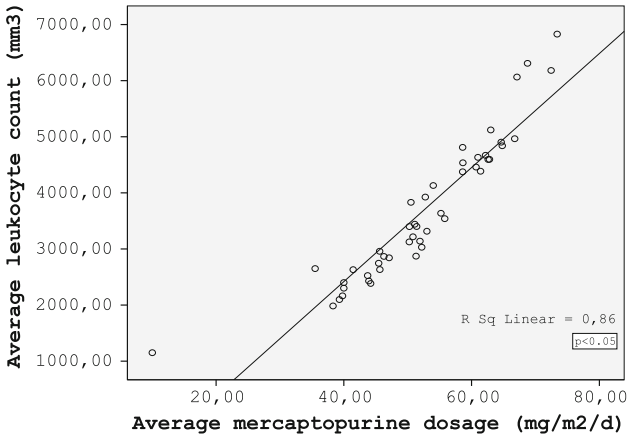

Fig. 1 Correlation between leukocyte count and mercaptopurine dosage

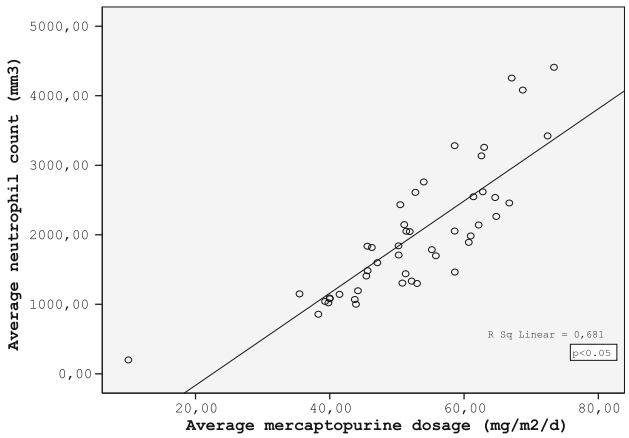

Fig. 2 Correlation between neutrophil count and mercaptopurine dosage

Table 2 Comparison of the toxicity between the mutant and non-mutant patients with leukemia during the first 24 weeks of maintenance chemotherapy with mercaptopurine

	Patients with mutation *n*: 4	Patients without mutation *n*: 47	*P* value
Age (years)	8.2 ± 4.6	7.2 ± 4.7	0.68
Gender (male/female)	2/2	31/16	0.44
White blood cell/mm^3 (mean ± SD)	2,125 ± 666	3,826 ± 1,209	0.008
Absolute neutrophil count/mm^3 (mean ± SD)	880 ± 454	2,086 ± 892	0.011
Percentage of 6-MP dosage (mg/m^2/day) (range)	62 (10–85)	108 (75–145)	0.000
Percentage of periods with infectious episodes graded ≥2	33	21	0.02
Percentage of periods with liver toxicity graded ≥2	31	16	0.01
Percentage of chemotherapy interruption	30	7	0.000
Percentage of periods with maintenance of leukocyte count <2,000/mm^3	46	2	0.000

chemotherapy interruptions were significantly higher than those without TPMT polymorphisms (Table 2).

Discussion

Previous studies have shown that TPMT*3A is the most common mutation occurring in 3.2–3.8% of Caucasian populations living in Europe and North America [11–14]. The frequency of TPMT*3C ranges between 0.2 and 1.0% while TPMT*3B and TPMT*2 are absent or very rare in these studies. In the present study, we have investigated the frequencies of these four TPMT mutations in an unselected group of Turkish children with ALL and found that 8.6% of children are heterozygous for a non-functional TPMT allele. The distribution of allele frequencies for TPMT*3A (3.4%) and *3C (0.9%) are similar to those of the Caucasian populations of Europe [11–13, 15]. These data are inconsistent with those of Tumer et al. who found the frequencies of each of TPMT*3A and *3C alleles being 0.9% in Turkish children with ALL [22]. The difference in the frequency of TPMT*3A between their study and ours might be due to the assays used to detect the polymorphisms or the composition or size of the study samples. In the detection of single nucleotide polymorphisms in DNA, the NonoChip (Electronic DNA microarray) technology used in our study is more sensitive than conventional PCR–RFLP in the study of Tumer et al. Higher frequency of TPMT*3A in our study group, which is a representative of the Turkish population, may not be explained by founder effect. Furthermore, our data are in good agreement with the results of a number of studies demonstrating that frequencies of mutations in several drug metabolizing enzymes such as CYP2D6, CYP1A1, and GSTM1/T1 in Turkish population is similar to other Caucasian populations [23, 24].

Individuals who are heterozygous for a non-functional TPMT variant have decreased enzyme activity and markedly increased levels of thioguanine nucleotides, which is the active metabolite of thiopurine drugs responsible for their antitumor activity. Intracellular thioguanine concentrations are inversely related to severity and duration of neutropenia during continuation therapy with 6-MP and methotrexate [25]. Relling et al. reported a fivefold greater cumulative incidence of 6-MP dose-limiting toxicity in children with ALL carrying variant TPMT during the maintenance therapy with daily 6-MP and weekly MTX of the St Jude Children's Hospital Total XIII Protocol. The cumulative incidence of 6-MP dose reduction due to toxicity was 100% in patients carrying two mutant alleles, 35% in patients with one mutant allele, and 7% in patients with wild-type TPMT [7]. Similarly, increased 6-MP toxicity was observed during maintenance therapy in TPMT*2, *3A, and *3B heterozygous children treated according to ALL-BFM-like protocols [14]. Although Stanulla et al. found no difference in hematopoietic and hepatic toxicity data between TPMT–wild-type and mutant heterozygous children during the ALL-BFM-2000 remission consolidation treatment, both the duration of chemotherapy was shorter (4 weeks vs. 24 weeks) and drugs used concomitant with 6-MP (intravenous cyclophosphamide and low dose cytosine arabinoside) were different in their study [26].

In an accord with the previous studies, our results showed that 6-MP therapy can be associated with increased hematopoietic and liver toxicity during maintenance therapy. The mean 6-MP dose in children carrying a variant TPMT allele (31 ± 14 mg/m^2/day) was about a half of those (54 ± 9 mg/m^2/day), used by children with wild-type TPMT. Children with a variant TPMT allele had more severe and profound leukopenia and neutropenia, more frequent neutropenic infections, grade ≥ 2 liver toxicity, and chemotherapy interruption as compared with the children with wild-type TPMT throughout the study period. These data lend further support to the suggestion that all children with ALL should be genotyped for common TPMT variants, preferably at the time of the diagnosis of leukemia for prospective adjustment of 6-MP dosage [14, 27]. This may allow appropriate administration maintenance chemotherapy which is important for the prognosis of leukemia [28].

Azathiopurine, another thiopurine drug, has been widely used in a number of autoimmune disorders and in solid organ transplantation [29–31]. Additionally, 6-thioguanine is frequently used in the treatment of acute myeloid leukemia in children [32]. Therefore, our results demonstrating that non-functional TPMT variants are not rare in Turkish population concerns a larger group of patients at high risk for severe bone marrow toxicity during treatment with thiopurine drugs. Determination of common TPMT variants can be cost-effective if increased infection rate and prolonged hospital stays or more frequent clinical visits due to excessive thiopurine toxicity is taken into account.

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