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Analysis ofThiopurineS-Methyltransferase Genotype in Children with Acute Lymphoblastic Leukemia by Strip Hybridization

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ABSTRACT:

*Acute lymphoblastic leukemia (ALL) is the most common pediatric cancer. The thiopurines, 6-mercaptopurine (6MP) and thioguanine (TG), are the backbone of current therapy for childhood ALL. Since their introduction to leukemia treatment in the 1950s, they have played an essential role in treatment protocols for ALL. ThiopurineS-methyltransferase (TPMT) polymorphism represents a determinant of 6-MP response and ALL outcome and is wellcharacterized in most populations. Four common polymorphic alleles areassociated with impaired activity of the enzyme. These are TPMT*2 (238G>C), TPMT*3A (460G>A, 719A>G), TPMT*3B (460G>A) and TPMT*3C (719A>G).*

Objective: *The aim of the present study was to determine the frequency of the functional TPMT polymorphisms and their association with the occurrence of adverse events, in pediatric patients with standard risk ALL who are subjected to 6-Mercatopurine therapy for consolidation.*

Patients and Methods: *TPMT polymorphism was analyzed in 40 children diagnosed with acute lymphoblastic leukemia and 40 age and sex matched healthy controls.The frequency of TPMT genotypes was examined by PXG-TPMT StripAssay based on Polymerase Chain Reaction (PCR) and reverse hybridizationusing blood samples. Clinical follow up using complete blood picture and liver*

transaminases following 6-MP therapy for consolidation were then performed for patients in order to access drug toxicity.

Results:*In the study sample, none had homozygous mutant TPMT genotypes (e.g. TPMT*3A/*3A, TPMT*2/*2, TPMT*3A/3C, etc.). Also neither the cases nor the controls in the study sample had TPMT*1/*2 and TPMT*1/*3B genotypes. In patients group, 39 (97.5%) were of the wild-type homozygous TPMT*1/*1 genotype, 1 (2.5%) patient only was of the heterozygous TPMT*1/*3A genotype and no patient had TPMT*1/*3C genotype. In the control group, we identified 36 subjects (90%) with wild-type homozygous TPMT*1/*1 genotype, 3 (7.5%) with heterozygous TPMT*1/*3A genotype and 1 (2.5%) heterozygous TPMT*1/*3C genotype. TPMT*3A was the most prevalent variant allele followed by TPMT*3C detected in the studied sample with an allelic frequency of 2.5% and 0.6%, respectively.The only patient with variant TPMT*1/*3A genotype did not show any evidence of thiopurine intolerance (hematotoxicity and hepatotoxicity).*

Conclusions: *Cases of myelosuppression in ALL pediatric patients treated with 6-MP cannot be all explained by the existence of TPMT alleles (*2, *3A, *3B and *3C). Other polymorphic alleles in TPMT gene or factors other than TPMT polymorphisms may be responsible for the development of toxicity.*

Key words:*TPMT polymorphism, thiopurine toxicity, pediatric ALL.*

INTRODUCTION

Pediatric ALL is one of the great success stories of modern cancer therapy, with contemporary treatment protocols achieving overall long-term event-free survival rate approaching 94%.[(1)]The thiopurines, 6-mercaptopurine (6MP) and thioguanine (TG), are the backbone of current therapy for childhood ALL. Since their introduction to leukemia treatment in the 1950s, they have played an essential role in treatment protocols for ALL. Several contemporary treatment protocols for childhood ALL apply consecutive cycles of either 6-mercaptopurine or thioguanine starting as early as during induction consolidation treatment and continue administration during maintenance therapy for up to 36 months after diagnosis.[(2)]

As pro-drugs, thiopurines require bioactivation by a multistep pathway to form thioguanine nucleotides, which are thought to be the major cytotoxic compounds through triggering cell cycle arrest and apoptosis. This process is in competition with direct inactivation of thiopurines or their metabolites by thiopurine S-methyltransferase (TPMT).[(2,3)]

TPMT is a cytosolic enzyme ubiquitously expressed in the human body and catalyzes the S-methylation of thiopurine drugs.[(2)] TPMT activity exhibits genetic polymorphisms in all large ethnic groups studied to date, including Caucasians, Africans, African- Americans, and Asians. These polymorphisms, which are inherited in an autosomal recessive trait, show a trimodal distribution in a Caucasian population resulting in 89% high, 11%

intermediate and 0.3% low or undetectable enzyme activity. Patients with intermediate activity are heterozygous at the TPMT gene locus, and the TPMT deficient subjects are homozygous for low activity alleles, as determined by molecular genetics and familial studies.[4-6]

The differences in TPMT activity result predominantly from single nucleotide polymorphisms (SNPs). The wild-type allele is designated as TPMT*1 and to date, at least 26 variant alleles of the TPMT gene have been identified. The prevalent alleles that have been associated with low or absent enzyme activity, are TPMT*2 (238G>C), TPMT*3A (460G>A, 719A>G), TPMT*3B (460G>A) and TPMT*3C (719A>G). These four alleles account for 80–95% of inherited TPMT deficiency and low enzyme activity.[7]

Several studies have investigated the prevalence of these variants in different populations. Among these four most common TPMT variants, TPMT*3C is the most prevalent mutant allele in African and Asian populations. In Egyptians, this mutant allele represents 86% of the TPMT variant allele.[8,9]

TPMT polymorphisms have been associated with the therapeutic efficacy and toxicity of thiopurine drugs. Numerous studies have shown that patients with inherited low levels of TPMT activity are at greatly increased risk for thiopurine-induced toxicity, when treated with standard doses of these drugs. These patients can be treated without major side effects (myelosuppression) when the dose is adjusted properly.[8-12]

Thiopurines are associated with two categories of adverse events. The first is the allergic type and the second is the non-allergic type. The first type usually occurs within the first 3-4 weeks of treatment and is not dose-dependent. It includes pancreatitis, fever, skin rash, general malaise and digestive intolerance. The second type seems to occur later; it is dose-dependent and includes hepatic toxicity, bone marrow toxicity, infections and malignancy.Myelosuppression is the most common serious complication.[7,12]

Since TPMT polymorphism represents a determinant of 6-MP response and ALL outcome, we aimed toinvestigate the relevance to introduce the prospective analysis of TPMT prior to any treatment, in order to individually optimize 6-MP therapy and avoid adverse reactions to this drug among the Egyptian children with standard risk ALL.

PATIENTS AND METHODS

This study was carried out on a total of 80 subjects divided into two groups. The first group included 40 pediatric patients with standard risk ALL who are subjected to 6-Mercatopurine therapy for consolidation while the second group included 40 age and sex matched controls.All the patients in our study were subjected to full history taking, thorough clinical examination and investigations. These investigations included complete blood picture, liver function tests, abdominal ultrasonography andbone marrow aspiration, cytochemistry (myeloperoxidase) and immunophenotyping by flowcytometry (for patients only) and TPMT

genotype detection by PXG - TPMT StripAssay based on Polymerase Chain Reaction (PCR) and reverse hybridization. Clinical follow up using complete blood picture and liver transaminases following 6-MP therapy for consolidation were then performed for patients in order toaccess drug toxicity.

Detection of TPMT genotype by PXG-TPMT StripAssay® based on PCR and reverse hybridization:

Two milliliters of venous blood was drawn into a sterile tube containing ethylenediamine tetra-acetic acid (EDTA) and stored at -20°C until the isolation of genomic DNA. Genomic DNA was extracted from EDTA whole blood samples by column method using a DNA extraction kit (QIAamp DNA Mini and QIAamp Blood Mini Kit from QIAGEN).The steps for DNA extraction included blood cell lysis, DNA binding, washes and elution. The TPMT genotype was then determined by PGX-TPMT StrioAssay® (ViennaLab Diagnostics GmbH, Austria) as follows: the extracted DNA was amplified using Polymerase Chain Reaction (PCR) technique using biotinylated primers specific for TPMT. After amplification, the amplified PCR products were subjected to agarose gel electrophoresis and were detected under ultraviolet light after staining with ethidium bromide as a quality assurance of amplification products. This was followed by hybridization of amplification products to a test strip containing allele-specific oligonucleotide probes immobilized as an array of parallel lines. Bound biotinylated sequences were detected using streptavidin-alkaline phosphatase and color substrates. The assay covered 3 polymorphic loci in the TPMT gene: 238 G>C, 460 G>A, 719 A>G.The PCR cycling protocol: Initial denaturation step at 94°C for 2 minutes, followed by 30 cycles of PCR, each of which consisted of denaturation at 94°C for 15seconds, annealing at 58°C for 30 seconds, extension at 72°C for 30 seconds and final extension step at 72°C for 3 minutes.

STATISTICAL ANALYSIS

The chi-square test was used for the comparison of the allele and genotype frequency between the cases and controls. The distribution of the genotype frequencies in both groups did not deviate from the Hardy-Weinberg equilibrium. A $P < 0.05$ was considered to be statistically significant. The SPSS statistical software package (version 18, SPSS, Chicago, IL, USA) was used for the statistical analysis.

DISCUSSION

In our study, genotyping of TPMT polymorphism using PXG-TPMT StripAssay based on PCR and reverse hybridization revealed that the homozygous wild-type TPMT*1/*1 was the most prevalent genotype in the study sample. No homozygous mutant TPMT genotypes were detected. Also neither the cases nor the controls in the study sample had TPMT*1/*2 and TPMT*1/*3B genotypes. In ALL patients, we found that 39 (97.5%) were of the homozygous wild-type TPMT*1/*1 genotype and 1 (2.5%) patient only was of the heterozygous TPMT*1/*3A genotype. In the control group, we identified 36 subjects (90%) with homozygous wild-

type TPMT*1/*1 genotype, 3 (7.5%) with heterozygous TPMT*1/*3A genotype and 1 (2.5%) heterozygous TPMT*1/*3C genotype. On statistically analyzing the difference in genotype distribution among the two groups, these differences did not reach statistical significance. All frequencies were in the Hardy-Weinberg equilibrium.

Regarding the prevalence of different TPMT variant alleles in the current study, we found that TPMT*3A is the most prevalent TPMT variant allele in the Egyptian children, accounting for 80% of the variant alleles detected. TPMT*3C is the second most recurrent variant and accounts for the remaining 20% of the TPMT variant alleles.

It is noteworthy to mention that the overall frequency of variant TPMT alleles in Egyptian children (3.13%) is lower than that reported for several Caucasian populations as British[8], Italian[13], German[6] populations and Ghanaians (7.6%)[8]but is compatible with that reported for Polish (3.2%)[14], Argentine (3,8%)[15], and Druze (3.9%)[16]. However, it is higher than the overall frequency reported in the Jordanian population (0.89%)[9], Tunisian population (0.84%)[17], Jews (0.75%)[16], Moslems (1.8%)[16] and some East Asian populations as Kazaks (1.2%)[18],and Taiwanese (0.6%)[19].

In agreement with our findings, Salah et al. reported that 117 of 119 healthy Tunisian individuals (98.3%) were homozygous wild-type TPMT*1/*1 genotype and only 2 subjects (1.68%) were heterozygous for TPMT*1/TPMT*3A genotype, with variant TPMT*3A allele being the only variant allele among subjects in their study.[17]

In contrast to our findings, several population studies revealed that the TPMT*3A allele is the most frequent mutant allele in Caucasians including, Americans, British, French, Germans, Italians, and Polish accounting for more than 80% of the variant alleles and that TPMT*3C is the most prevalent variant allele in African and Asian populations accounting for 100% in Japanese, Malaysian and Ghanaian, 70% of African-Americans and 5% only in Caucasians.[6-24]

The discrepancy between our results as a whole and those of other studies may be explained by the low proportion of eligible cases and controls included in the analysis, small sample size, difference in study design, the selection of the population studied, and gene-environment interactions, such as diet, exposure to chemicals and drugs.

Thiopurine Intolerance

Further, the present study also investigated the association between different TPMT genotypes and the occurrence of thiopurine intolerance following the consolidation chemotherapy. It was demonstrated that only one ALL patient had heterozygous TPMT*1/*3A genotype while the remaining 39 patients had wild type TPMT*1/*1 genotype. This patient did not develop any sign of 6-MP toxicity after the continuation of 4 weeks of consolidation chemotherapy. His CBC showed a WBC of 4.5 $x10^9$/L, absolute neutrophil count of 3375.0/µL and platelet count of 519.0 $x10^9$/L. He had an elevated ALT result which was less than two-fold increase (ALT= 110 U/L) and his AST result was slightly elevated (AST= 49 U/L). He also

achieved complete remission as assessed by a bone marrow aspirate withdrawn on day 28 ± 1 after initiation of consolidation chemotherapy having 3% blast cells in a normocellular bone marrow aspirate.

Correlations between the different TPMT genotypes in ALL patients regarding their clinical picture, sex, development of thiopurine intolerance (myelosuppression and/ or hepatotoxicity) were not applicable as only one case had a variant TPMT genotype .

In contrast to our findings, several studies found an association between the presence of the variant TPMT genotypes in pediatric ALL patients and the occurrence thiopurine intolerance. Aboul Naga et al. and others reported that TPMT mutant patients, especially homozygous, were at greater risk of 6-MP toxic effects and needed more frequent dose reductions which emphasizes the importance to introduce the prospective analysis of TPMT genotype, prior to any ALL treatment.[4,24-27]

The lack of association between variant TPMT genotype and occurrence of thiopurine intolerance in our study may be explained by the timing of assessment for toxicities as most studies assessed the occurrence of toxicities during maintenance therapy for ALL patients after being exposed to the drug for a longer period. Also, the small cohort size, particularly number of patients with mutant TPMT genotype and inclusion and exclusion criteria adopted in different studies may have been other contributory factors .

It is also important to note that in the sample population of the present study, 13 0f 39 ALL patients with TPMT*1/*1 genotype (33.3%) developed thiopurine intolerance after the continuation of consolidation chemotherapy in the form of myelosuppression alone with no hepatotxicity.

In accordance with our findings, Tantawy et al. and Ayesh et al. reported that genetic polymorphism in TPMT is not one of the determinant causes of 6-MP toxicity in ALL patients. These studies reported that the most important causes of dose modification and interruption were environmental factors (infections and febrile neutropenia).[28,29]

The presence of thiopurine intolerance among ALL patients with wild-type TPMT*1/*1 genotype may indicate the presence of either rare mutations in TPMT gene or additional SNPs in genes encoding enzymes involved in 6-MP metabolism and transport that might contribute to the drug-induced toxicity ; a finding that was supported by several studies investigating the clinical relevance of SNPs in these genes in ALL patients including genetic polymorphisms in ITPA enzyme, which catalyzes one of the intermediate steps of 6-MP metabolismor in MTHFR enzyme, probably because of their impact on S-adenosylmethionine, which functions as a cofactor for TPMT. Other polymorphisms in genes involved in 6-MP disposition such as xanthine oxidase (XO) or ATP-binding cassette sub-family C member 4 (ABCC4) have also been suggested. Also, non-genetic factors as diet and drug interactions may be implicated to our finding. Thus, the adverse effects caused by the thiopurine treatment is said to be attributable to multi-factorial phenomena involving multiple biological and environmental processes (including drug

interactions), other than the mutated genotype alone.[12,30-37]

In conclusion, TPMT*3A was the most prevalentvariant allelefollowed by TPMT*3C detected in the Egyptian pediatric ALL patients with an allelic frequency of 2.5% and 0.6%, respectively. Cases of myelosuppression in ALL pediatric patients treated with 6-MP cannot be all explained by the existence of TPMT alleles (*2, *3A, *3B and *3C). Other polymorphic alleles in TPMT gene, or factors other than TPMT polymorphisms may be responsible for the development of toxicity.

Table (1): Distribution of ALL patients and controls according to TPMT genotype and TPMT allele frequencies

	Cases		Control		Total	
	No.	%	No.	%	No.	%
TPMT genotype						
*1/*1	39	97.5	36	90.0	75	93.8
*1/*3A	1	2.5	3	7.5	4	5.0
*1/*3C	0	0	1	2.5	1	1.2
*1/*2	0	0	0	0	0	0
*1/*3B	0	0	0	0	0	0
P	0.346					
TPMT allele						
TPMT*1	79	98.75	76	95	155	96.9
TPMT*3A	1	1.25	3	3.75	4	2.5
TPMT*3C	0	0	1	1.25	1	0.6
TPMT*3B	0	0	0	0	0	0
TPMT*3C	0	0	0	0	0	0
P	0.373					

p: p value for Monte Carlo test for comparing between the two studied group

*: Statistically significant at $p \leq 0.05$

RESULTS

In this study, the sample included 40 pediatric patients with standard risk ALL and 40 age and sex matched controls. The patient group included 23 males and 17 females whereas the control group included 23 males and 17 females. 21 patients were in the ≤5 year old age group and 19 patients were in the >5 year old age group. On the other hand, 14 of the control group were in the ≤5 year old age group and 26 were in the >5 year old age group.

The genotyping of the TPMT polymorphism:

Genotyping of the TPMT polymorphisms using PXG – TPMT StripAssay based on PCR and reverse hybridization was successful in all 80 subjects.In the study sample, none had homozygous mutant TPMT genotypes (e.g. TPMT*3A/*3A, TPMT*2/*2, TPMT*3A/3C, etc.). Also neither the cases nor the controls in the study sample had TPMT*1/*2 and TPMT*1/*3B genotypes. In patients group, 39 (97.5%) were of the wild-type homozygous TPMT*1/*1 genotype, 1 (2.5%) patient only was of the heterozygous TPMT*1/*3A genotype and no patient had TPMT*1/*3C genotype. In the control group, we identified 36 subjects (90%) with wild-type homozygous TPMT*1/*1 genotype, 3 (7.5%) with heterozygous TPMT*1/*3A genotype and 1 (2.5%) heterozygous TPMT*1/*3C genotype. TPMT*3A was the most prevalent variant allele followed by TPMT*3C detected in the studied sample with an allelic frequency of 2.5% and 0.6%, respectively (Table 1). No statistically significant differences were found between patient and control groups.

Incidence of thiopurine intolerance in ALL patients with different TPMT genotypes

After continuation of 4 weeks of consolidation chemotherapy, the only patient with variant TPMT*1/*3A genotype did not show any evidence of thiopurine intolerance (hematotoxicity and hepatotoxicity). On the other hand, 13 0f 39 ALL patients with TPMT*1/*1 genotype (33.3%) developed thiopurine intolerance after the continuation of consolidation chemotherapy in the form of myelosuppression alone with no hepatotxicity. Six of 11 patients developed leucopenia and neutropenia, 5 patients developed neutropenia alone, 1 patient developed leucopenia alone and the remaining patient developed leucopenia, neutropenia and thrombocytopenia as well as failing to achieve complete remission (7% blast cells in a hypocellular bone marrow aspirate).

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