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Passive smoking and thiopurine methyl transferase genotyping in 6-mercaptopurine-induced myelosuppression in Egyptian children with acute lymphoblastic leukemia

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Abstract---6-mercaptopurine (6-MP) is a crucial component drug used to treat childhood acute lymphoblastic leukemia (ALL) in the maintenance phase. Thiopurine S-methyltransferase (TPMT) is an enzyme that metabolizes thiopurine drugs like 6-MP. In patients taking thiopurine medications, lack of TPMT activity is linked to an increased risk of myelosuppression. Patients with TPMT polymorphisms are more likely to develop mercaptopurine-induced myelosuppression. The goal of this study was to see how passive smoking and TPMT genetic variations affected 6-MP-induced myelosuppression in Egyptian ALL children. This study included 100 children: 50 with acute lymphoblastic leukemia in the maintenance phase of treatment who had a history of passive smoking and 50 age-matched healthy controls. 6-MP was given to patients in the maintenance phase, and myelosuppression was monitored at the end of this phase by comparing WBC counts to those obtained at the start of treatment. After six months of 6-MP administration, there was no significant association between cotinine levels and WBC count (as evidence of myelosuppression), although there was a significant correlation between TPMT genotypes and WBC count, with 75% of hetero genotype patients suffering from severe declines in WBC count

and 91% of wild genotype people undergoing mild to moderate decreases in WBC count.

Keywords---passive smoking, thiopurine methyl transferase, 6-mercaptopurine, acute lymphoblastic leukemia, myelosuppression.

Introduction

Leukemia is a group of hematological illnesses characterized by abnormal lymphocyte growth and proliferation. They are classified as acute or chronic depending on the degree of cell differentiation, as well as myelocytic or lymphocytic depending on the predominant cell type. Although several genetic and environmental risk factors have been identified, the exact cause of most leukemia subtypes remains unknown (Lyengar & Shimanovsky, 2021). Smoking is a leading cause of cancer and immune-mediated inflammatory disorders. Tobacco smoke comprises a complex mixture of chemicals, including a variety of reactive oxygen and nitrogen species (ROS and RNS), which can harm lipids, proteins, and nucleic acids at the cellular and subcellular levels. A growing body of evidence supports a key role for smoking-induced ROS and the resulting oxidative stress in inflammation and carcinogenesis. Human carcinogens such as benzene, formaldehyde, butadiene, polycyclic aromatic hydrocarbons, and polonium are found in both mainstream and side stream cigarette smoke. Benzene is known to harm hematopoietic stem cells, making it a possible contributor to the development of paediatric ALL (Cogliano *et al.*, 2011).

Treatments for all types of leukemia are improving, allowing people to live longer and more fully with this disease. The ALL treatment approach is one of the most complex and rigorous cancer treatment strategies. While the specific treatment regimens and drug selection, dosing schedules, and therapy periods vary from patient to patient, the core treatment concepts remain consistent. In general, the therapy phases can be divided into three categories: induction, consolidation, and maintenance. 6-mercaptopurine (6-MP) is one of the most frequently prescribed chemotherapy drugs in the treatment of ALL (Dresden & Ranchod, 2021). Mercaptopurine is an immunosuppressant and anti-neoplastic agent that belongs to the thiopurine class of drugs. It is used in combination with other drugs to treat acute lymphoblastic leukemia, the most common type of cancer in children. The inclusion of 6-TGTP into DNA is partly responsible for mercaptopurine cytotoxicity. Mercaptopurine is inactivated in two ways: by methylation, which is catalyzed by TPMT, and through oxidation, which is catalyzed by xanthine oxidase (XO). The response to 6-MP and other purine analogs is known to be affected by genetic variation in the activity of enzymes involved in the purine salvage pathway. While hepatotoxicity and other GI side effects with 6-MP are infrequent, they can cause delays in chemotherapy, reduced 6-MP dosing, and difficult lifestyle modifications, all of which can significantly impact a child's quality of life while also increasing their chance of relapse (Dean & Kane, 2020)(Conneely *et al.*, 2020). The primary purpose of the present study was to see how passive smoking and thiopurine methyl transferase genotyping affected white blood cell count as a toxicity related marker of 6-mercaptopurine in Egyptian children with acute lymphoblastic leukemia.

Materials and Methods

Patients and controls

This study included fifty ALL children with a history of passive smoking who were diagnosed using standard clinical, morphological, cytochemical, and immunophenotypic criteria and treated using the St. Jude ALL Total Therapy Study XV treatment protocol (Pui *et al.*, 2009) at the Pediatric Oncology Unit, National Cancer Institute, Egypt from September 2019 to August 2021, as well as fifty healthy children who did not complain of any illness. Patients with Down syndrome, those on other therapy regimens, those who did not achieve remission after induction, those who relapsed or died before commencing the maintenance phase of treatment, and those with impaired renal or hepatic functions upon diagnosis were excluded from the current study. The study was approved by Ethical Committee of the National Research Centre. Informed consent was obtained from the parents of both patients and control children before starting of the study.

Sample collection

Samples were collected from both controls and patients and were processed using serial numbers and stored until analysis. The samples were taken from the patients at the beginning of maintenance therapy with 6-MP and after six months. The rationale for completing maintenance therapy after six months was to allow enough time for toxicity monitoring (Peregud-Pogorzelski *et al.*, 2011). Sample collection was carried out as follows: at the start of maintenance therapy, for the assessment of the cotinine level in serum as a biomarker of passive smoking using the ELISA method; TPMT gene genotyping via PCR-sequencing for variation detection; and white blood cell (WBC) count as a baseline for comparison six months after starting maintenance therapy using an automated hematology analyzer. WBC were re-counted after six months of maintenance medication to determine bone marrow affection caused by 6-MP-related toxicity.

Methods

Measurement of cotinine: measurement of serum cotinine level was carried out using Enzyme-Linked Immunosorbent Assay (ELISA) method. The samples and cotinine enzyme conjugate was added to the wells coated with anti-cotinine antibody. Cotinine in the samples competes with a cotinine enzyme conjugate for binding sites. Unbound cotinine and cotinine enzyme conjugate is washed off by washing step. Upon the addition of the substrate, the intensity of color is inversely proportional to the concentration of cotinine in the samples. A standard curve is prepared relating color intensity to the concentration of the cotinine (Wielkoszynski *et al.*, 2009).

Thiopurine methyltransferase (TPMT) genotyping: Thiopurine methyltransferase genotyping was determined by PCR-sequencing for variation detection. PCR amplification and enzymatic digestion of the products was performed by PCR-restriction fragment length polymorphism analysis to evaluate genetic polymorphism in TPMT (TPMT*3B [G460A], TPMT*3C [A719G], and TPMT*3A

[G460A and A719G]]. For G460A polymorphism, DNA at exon 7 was amplified with a forward primer 5,CAGGCTTTAGCATAATTTTCAATTCCTT-3, and reverse primer 5,TGTTGGGATTACAGGTGTGAGCCAC-3, While at exon 10 for A719G polymorphism, the forward primer was 5-CTT TGG GGA GCT GAA GGA CTA CTA C-3), and the reverse primer was 5-CAC TTT GTG ACC ATT CCG GTT TG-3. After amplification, the PCR products were digested by AccI Fast Digest restriction enzyme for 719A > G at exon 10 and by MwoI FastDigest restriction enzyme for 460G > A at exon 7. The digestion products were separated by agarose gel electrophoresis and were detected under ultraviolet light after staining with ethidium bromide (El-Rashedy *et al.*, 2015).

White Blood cells Count (CBC): WBC count was done using Coulter DxH 500 SERIES HEMATOLOGY ANALYZER. The Coulter DxH 500 is an electronic method for counting and sizing particles. Although the Coulter Principle can be used to calculate and size just about any particle, the specific application of this principle in hematology is to count and size the White Blood Cells (Coulter, 1956). Statistical analysis was performed using the R-statistical program version (4.0.4). When comparing a numeric variable between two independent and dependent groups, an independent T test (T) or paired T test (pT) was used. If parametric assumptions were met, then variables were described as mean $\pm$ std. In the case of parametric assumption violence, Manwhitney (M) or Willcoxon (W) tests were used in the case of independent or dependent groups, respectively. And variables were then described using the median [interquartile range]. Fischer's Exact Test (F) was used to test the correlation between two categorical variables and the expression of these variables as count (percentage). A P-Value of less than 0.05 was considered significant in all tests.

Results

The parameters of the current investigation were determined using fifty patients with acute lymphoblastic leukemia (ALL) and fifty healthy children (as controls). The patients were assessed before receiving 6-MP therapy and their parameters were referred to as before treatment. Then they were re-examined six months later and their post-treatment parameters were referred to as after treatment.

Serum cotinine levels (ng/ml) among patients before treatment with 6-MP drug and control group

The serum cotinine levels were measured in patients before treatment with the 6-MP drug and the control group. The results are demonstrated in table 1. In the control group, the median was 1.5 (ng/ml), with an interquartile range of 0.9-3.5 (ng/ml). While in the patient group before treatment, the median was 6.15 (ng/ml), with an interquartile range of 4.8-7.5 (ng/ml). Compared to the two groups, the cotinine level was much higher in the patient group than in the control group. These data showed that there was a highly significant difference (P-Value <0.0001) between the two groups (Fig. 1) Table 1, which indicates that the patient group has been exposed to passive smoking.

Distribution of TPMT genotype (%) among patients and control group

The TPMT genotype distribution in patients and controls was studied. As shown in table 4, 8% (4/50) of patients have hetero type, which is an aberrant genetic variant, whereas 100% (50/50) of subjects in the control group have wild type (normal type). This difference, however, was not statistically significant (P-Value > 0.05 Figure 2 table 1).

WBCs Count ($\times 10^3 \mu\text{L}$) among patients before treatment with 6-MP drug and control group

The WBC count in patients before treatment with the 6-MP drug and in the control, group was estimated. Before treatment, the median for the patient group was $4.1 (\times 10^3 \mu\text{L})$, with an interquartile range of $3.62\text{--}5.2 (\times 10^3 \mu\text{L})$. The control group's median was $6.7 (\times 10^3 \mu\text{L})$, with an interquartile range of $5.8\text{--}8.3 (\times 10^3 \mu\text{L})$, as shown in Figure 3 table 1. The difference between the two groups was highly statistically significant (P-Value < 0.0001), demonstrating the effect of medication on WBC count during the induction and consolidation phases of treatment, as it reduced WBC count to the lowest normal level, but not to the level of leucopenia, when compared to the control group.

Table 1

The correlations of measured parameters between the patients before treatment with 6-MP drug and the control group

Parameter	Control	Patients before treatment	P-Value
Cotinine (ng/ml serum)	1.5 [0.9-3.5]	6.15 [4.8-7.5]	<0.000 (^M)
TPMT genotype (Hetero)	0(0%)	4(8%)	0.117(^F)
WBCs ($\times 10^3 \mu\text{L}$)	6.7[5.7-8.3]	4.1[3.62-5.2]	<0.000 (^M)

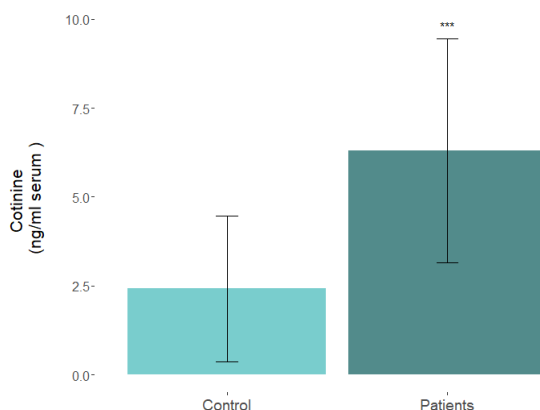


Figure 1. Cotinine level (ng/ml) among patients before treatment with 6-MP drug and control group

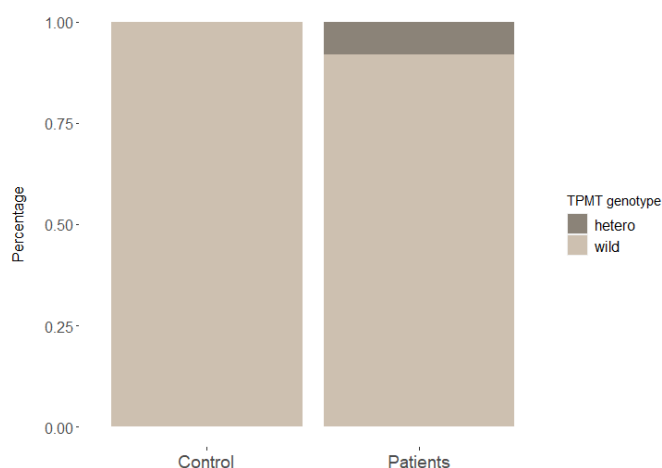


Figure 2. Distribution of TPMT genotype (%) among patients and control group

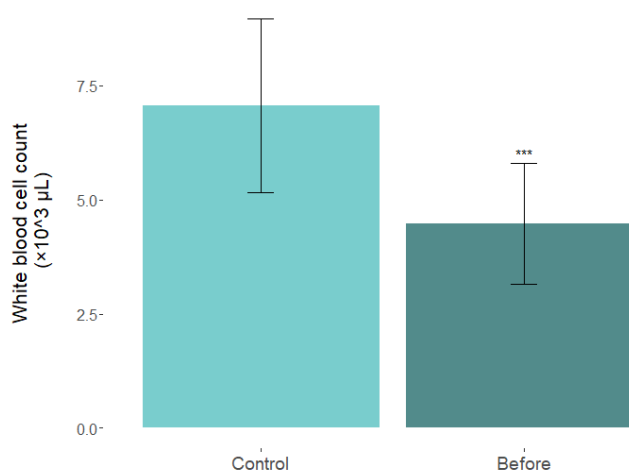


Figure 3. WBCs count among patients before treatment with 6-MP drug and control group

Patients' WBC count before and after six months of administration of 6-MP drug

The WBC count of patients before and after six months of administration of 6-MP was monitored. As shown in table 2, the median of the patient group before treatment was $4.1 (\times 10^3 \mu\text{L})$ with an interquartile range of $3.6-5.2 (\times 10^3 \mu\text{L})$, and the median of the patient group after treatment was $3.55 (\times 10^3 \mu\text{L})$ with an interquartile range of $2.6-3.8 (\times 10^3 \mu\text{L})$. Figure 4 shows that the difference between these two groups was highly significant (P-Value 0.0001). The WBCs count was affected as a related marker of 6-MP toxicity, which was meant to recover to normal following treatment, but the WBCs count continued to decline, indicating myelosuppression and the need to adjust the medication dose to reduce the related toxicity.

Table 2
WBCs count ($\times 10^3 \mu\text{L}$) of patients before and after six months of administration of the 6-MP drug

Parameter	Before	After	P-Value
WBCs ($\times 10^3 \mu\text{L}$)	4.1[3.6-5.2]	3.55[2.6-3.8]	<0.000 ^(W)

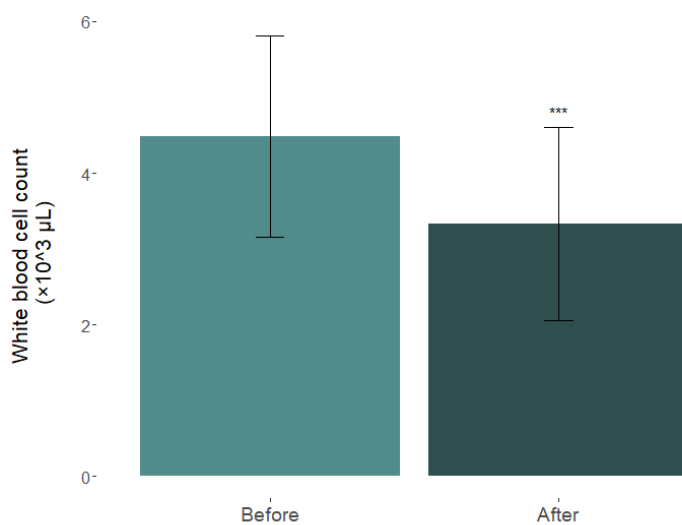


Figure 4. WBCs count of patients before and after 6 Months administration of 6-MP.

Table 3
Frequency of WBC count among patients before and after six months of administration of the 6-MP drug and the control group

Parameter		Categories	Score	Frequency		
				Control	Before	After
WBCs	Normal	>4	0	48	27	5
	Mild	3-4	1	2	20	24
	Moderate	2-3	2	0	2	14
	Severe	<2	3	0	1	7

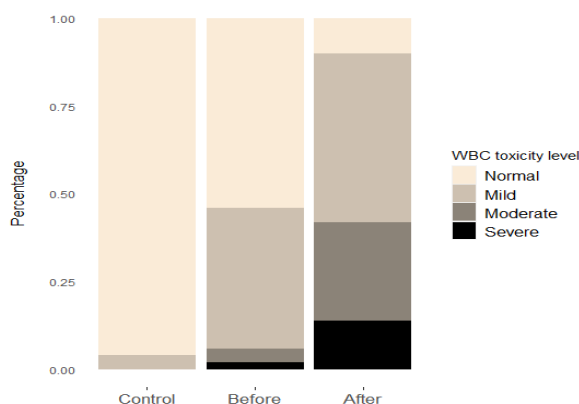


Figure 5. The frequency of WBC count among patients before and after six months of administration of the 6-MP drug and the control group

Compared toxicity with cotinine in patients after treatment with 6-MP

The correlation between cotinine level and the WBCs count as a toxicity related marker of 6-MP drug in patients after six months of administration was studied as shown in Table (4). 56 % (20 / 50) of the patients with mild leucopenia , 22% (8 / 50) with moderate leucopenia and 20% (5 / 50) with sever leucopenia. Whereas all correlations were not statistically significant as revealed in figure 6.

Table 4

Correlation between different cotinine levels and degree of WBCs as a toxicity marker after six months administration of the 6-MP drug

WBCs					0.286 ^(F)
	Normal	Mild	Moderate	Severe	
Normal	2(14%)	4(29%)	6(43%)	14(8%)	
Passive smoker	3(8%)	20(56%)	8(22%)	5(20%)	

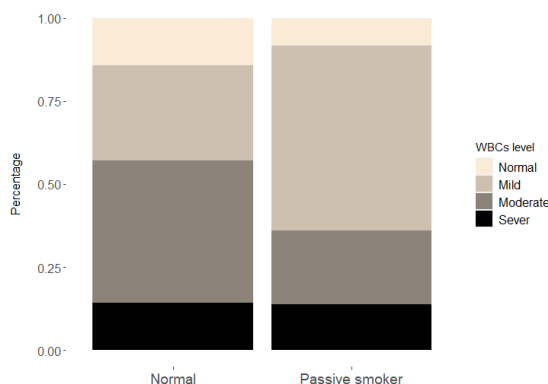


Figure 6. Distribution of different WBCs count after six months administration of 6-MP among different cotinine-levels groups

Compared toxicity with TPMT genotype in patients after treatment with 6-MP drug

The correlation between TPMT genotype and WBC count were studied. Where 75% (3 / 50) of Hetero genotype patient suffered from severe decline in WBCs whereas most of patients with wild genotype 50% (23 / 50) , 30% (14 / 50) suffered from mild and moderate decrease in WBCs, respectively and Only 9% (4 / 50) suffered from severe WBCs as in table 5 figure 7

Table 5
The correlation between different TPMT Genotypes and degree of WBCs Toxicity after 6 Months administration of 6-MP

WBCs					
	Normal	Mild	Moderate	Severe	
Wild	5(11%)	23(50%)	14(30%)	4(9%)	0.01 ^(F)
Hetero	0(0%)	1(2%)	0(0%)	3(75%)	

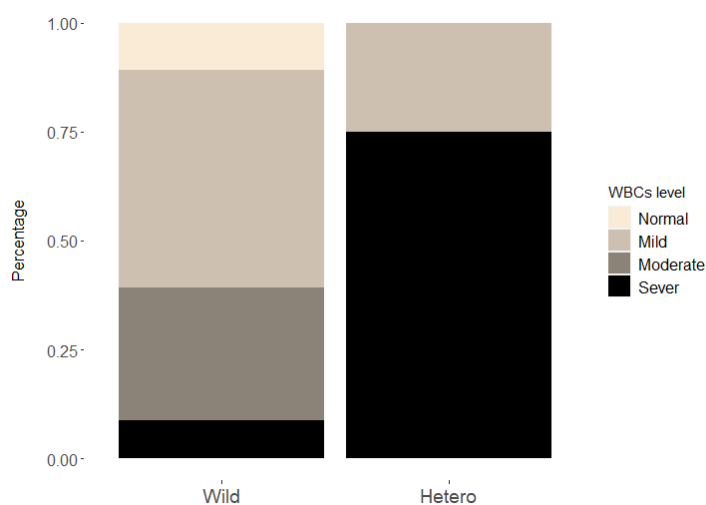


Figure 7. Distribution of different level of toxicity of WBCs after six months administration of 6-MP drug among different genotypes

Discussion

In this study, the correlation between cotinine level and WBCs count (as a related toxicity marker) after six months of 6-MP administration was studied, and it was found that the correlation was non-significant. These findings contradicted those of Cárceles-Ivarez *et al.* (2019), who discovered that secondhand smoking (SHS) reduces overall survival and increases the incidence of treatment-related mortality in children with ALL. The amount of cigarettes smoked by parents had no effect on oxidative stress, suggesting that an individual's sensitivity to tobacco's effects is independent of parental intake. The effects of tobacco exposure on the survival of children with ALL could be explained in multiple ways. Firstly, previous studies

have associated prenatal exposure to tobacco smoke to the presence of leukemogenic cytogenetic alterations with worse prognosis (such as the MLL rearrangement) in offspring (de Smith *et al.*, 2017) and DNA methylation patterns, that also worsen the prognosis in childhood ALL (Borssén *et al.*, 2018). Secondly, passive smoking increases the frequency and severity of infections in children. Infections are the leading cause of treatment-related mortality in childhood ALL. This is due to nicotine reduces the phagocytic activity of macrophages, inhibits Th-1 helper cells which are responsible for the production of IgG, and stimulates Th-2 helper cells, which increase cytokines and interleukins, resulting in a chronic inflammatory response. Furthermore, nicotine inhibits natural killer cells, reduces cellular cytotoxic activity, affects the mucociliary epithelium by direct toxic injury, and enhances pathogenic bacterium adherence (Orgel & Bhojwani, 2017).

Another possible mechanism would be to stimulate tumor progression. Both nicotine and the activation of nicotinic acetylcholine receptors (nAChR) can increase tumor proliferation, migration and invasion of other organs, angiogenesis, and epithelium-mesenchyme transition (Schuller, 2012). Furthermore, nicotine can reduce the efficacy of chemotherapy and radiation therapy, as well as enhance the toxicity and side effects of chemotherapy (Gritz *et al.*, 2005). Children may be exposed to passive smoking in the form of third-hand smoke (THS) due to residues that remain in the home and are deposited on surfaces. THS exposure pathways can occur through inhalation, dermal uptake from contact with contaminated surfaces, and ingestion of THS found on hands or in food. Toddlers may be exposed to THS through mouthing things in their environment. The duration of THS inside will typically be significantly longer than that of second-hand smoke, and could last for months (Jacob *et al.*, 2017).

In this study the correlations between TPMT genotype and WBCs count, were studied and all correlations was significant WBCs count (evidence of myelosuppression). Regarding WBCs count, 75 percent of heterozygous genotype patients suffered from severe decline in WBCs count, whereas most of patients with wild genotype experienced mild and moderate decrease in WBCs count and only 9 percent of them suffered from severe reduction in WBCs count. These results were consistent with those by El-Rashedy *et al.* (2015), who revealed that high frequency of *TPMT* mutant alleles among the studied ALL children with a unique pattern. *TPMT* polymorphism increased the risk for 6-MP related hematological toxicity and its fatal outcome that was more prominent in the homozygous form. This emphasizes the importance of common *TPMT* variants determination in ALL children prior to therapy which could help tailoring 6-MP doses and reducing the associated toxicity.

Also Jantararoungtong *et al.* (2021) found that genetic variations in genes encoding drug-metabolizing enzymes and drug transporters can influence the response to 6-MP. The dosage of 6-MP and its adverse effects were assessed from medical records at 24 weeks of maintenance therapy. Children with the *TPMT* heterozygotes genotype had a higher risk of leukopenia compared to wild type patients. Severe neutropenia was shown to be related with heterozygous *TPMT* patients. The evidence that *TPMT* heterozygotes are associated with increased of 6-MP-induced myelotoxicity also backs up the compelling need for genotype this

pharmacogenetic marker prior to starting 6-MP medication. A study by Kouwenberg *et al.* (2020) suggested that before starting ALL maintenance treatment, the TPMT genotype should be considered as part of standard examination.

Wild-type genotypes are the most common with no evidence of 6-MP related toxicity, whereas those with mutant genotypes sometimes displayed symptoms of 6-MP drug toxicity. Myelosuppression and/or hepatotoxicity were reported in some patients with wild-type *TPMT*. At diagnosis, the only mutant patient genotype showed leukopenia, absolute neutropenia, thrombocytopenia, and elevated ALT level; however, all of these symptoms recovered with therapy, except for ALT. The presence of these abnormal results at diagnosis was mostly due to lymphoblast infiltration of the bone marrow and/or extramedullary organs. However, after therapy, the abnormal CBC findings and abnormal liver functions in those patients might be due to increased thioguanine nucleotides (TGNs) inside the cells and decreased activity of TPMT enzyme despite absence of polymorphism. Infections or any other unknown environmental factors unrelated to 6-MP treatment could also be to blame for these abnormal laboratory findings (El-kaffash *et al.*, 2014). Adam de Beaumais *et al.* (2011) reported that the genotype of TPMT can be determined before therapy to identify homozygote deficient patients who are at high risk of severe and life-threatening toxicity. Intracellular 6-MP metabolites will become helpful markers to monitor 6-MP during ALL maintenance therapy, in addition to white blood cell count. Individual demographic and pharmacogenetic markers linked to pharmacokinetics will allow the individualization of drug administration and improve the outcome of ALL pediatric patients.

Conclusion

This study found that TPMT polymorphism increased the risk of 6-MP-related hematological toxicity and myelosuppression, with the most severe outcome occurring in the heterozygous form, highlighting the importance of defining common TPMT variants in ALL children prior to therapeutic interventions with 6-MP drugs, which could help adjust 6-MP doses and reduce associated toxicity. Furthermore, the current findings indicate that the history of passive smoking had no influence on 6-MP-induced myelosuppression.

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