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Detection of Thiopurine S-Methyltransferase (TPMT) Polymorphisms TPMT*3A, TPMT*3B and TPMT*3C in Children with Acute Lymphoblastic Leukemia

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Abstract:

Background: Thiopurines are essential medications in Acute Lymphoblastic Leukemia (ALL) treatment protocols as anti-cancer agents since long time; however, their use might result in unexpected toxicities in ALL children due to the low thiopurine S-methyltransferase (TPMT) activity, a major enzyme involved in 6- mercaptopurine metabolism, which strongly correlates to the genetic polymorphism of the TPMT gene in those patients.

Objective: To identify the most common TPMT polymorphisms in children with ALL and its frequencies.

Methods: A cross sectional study enrolling eighty-one ALL children receiving mercaptopurine drug during their maintenance course of treatment according to UKALL – 2011 protocol, were enrolled in this study. After DNA extraction from whole blood TPMT genetic polymorphisms were detected by allele-specific multiplex-PCR analysis.

Results: A total of 51 children with allele frequencies of (62.96%) were homozygous for the wild-type allele TPMT*1, 30 children with allelic frequency of (37.03%) were heterozygous for one of the two mutant alleles (TPMT*3A or TPMT*3C) with allele frequencies of 29.62% and 7.4% respectively, while no result was found homozygous for two mutant alleles or TPMT*3B allele.

Conclusions: This is the first study in Iraq to identify the genetic polymorphism of TPMT in a group of ALL children being treated for ALL. The study revealed the presence of TPMT*3A and TPMT*3C genetic polymorphisms among the study sample, no TPMT*3B was identified in the study sample.

Keywords Acute Lymphoblastic Leukemia, 6-Mercaptopurine, Thiopurine S-methyltransferase, genetic polymorphisms.

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Introduction:

Extraordinary advances in the treatment outcome of childhood Acute Lymphoblastic Leukemia (ALL) rank as one of the most successful stories in the history of oncology, with the current rate of approximately 80% of children being cured [1,2]. In spite of this success, there remains place for improvement, including the development of less toxic therapy [3, 4]. Thiopurine drugs, one of the medications that plays a major role in the maintenance phase of treatment of ALL, these drugs are purine analogue drugs which are metabolized inside the human body to form thioguanine nucleotide metabolites (TGNs), which have cytotoxic and immunosuppressive properties. However, these drugs like many cytotoxic agents have a relatively narrow therapeutic index. 6-Mercaptopurine (6-MP) is taken orally daily for a considerable period of time during the maintenance course of ALL treatment which last for 2-3 years [5]. Plasma concentrations peak are reached after 1–2 hr in most patients following oral intake of 6-MP and then rapidly decline with half-lives of less than 1 hr [6]. In particular, thiopurine S-methyltransferase enzyme is responsible for the inactivation of antimetabolite thiopurine drugs, and it is affected by the sequence variations in the TPMT gene [7]. TPMT is a polymorphic enzyme and till now around 38 TPMT variant alleles have been discovered, most of them are single nucleotide polymorphisms (SNPs) that resulting in an amino acid change [8]. Although ALL treatment is successful in many aspects, but up to 25% of children suffer from severe side effects such as myelosuppression which are due to low TPMT activity that result from TMPT gene polymorphisms when treated with standard doses of 6-MP [9]. TPMT activity exhibits monogenic co-dominant inheritance polymorphism in all large ethnic groups. The 90% of population is homozygous for the wild type allele (TPMT*1) with high TPMT enzyme activity, One in 300 persons have homozygous for a mutant allele with very low TPMT activity leading to severe toxicity [10], 10%

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of population has heterozygous for one mutant allele with intermediate enzyme activity, leading to moderate myelosuppression when those patients are treated with standard dose [11]. Three SNPs characterize the four defective alleles that account for more than 95% of polymorphisms, TPMT * 2, TPMT*3A, TPMT*3B and TPMT*3C while the wild-type has been designated as TPMT*1. The detailed changes of these alleles are:

- TPMT * 2 allele contains single 238 G > C mutation (transversion),
- TPMT * 3A allele has 2 mutations (460 G > A and 719 A > G)
- TPMT * 3B allele contains single 460 G > A mutation (transition)
- TPMT * 3C has only 719 A > G mutation (transition) [12]

There is no information available for the Iraqi population TPMT polymorphisms. Therefore, the aim of this study was to detect the most common TPMT allele polymorphism frequencies (TPMT*3A, TPMT*3B and TPMT*3C) in a sample of Iraqi children with ALL diagnosis during their maintenance course therapy under 6-MP drug therapy (the children were attending the hematology/oncology outpatient clinic of Children Welfare Teaching Hospital in Baghdad Medical City).

Subjects and Methods:

Subjects: Eighty-one pediatric patients with a diagnosis of ALL during their maintenance course attending the hematology/oncology outpatient (OP) clinic of Children Welfare Teaching Hospital (CWTH) in Baghdad Medical City during the period between March 2017 to July 2017 were enrolled in this study. Those patients were receiving 6-MP drug as part of their treatment plan according to the guidelines of UKALL 2011 protocol (United Kingdom Acute Lymphoblastic Leukemia). The oncology unit of CWTH receives and treats patients below the age of 14 years. Subjects' information that includes age, gender, body weight, and surface area of the child were recorded. Medical information of each patient such as the subtype of ALL and the time period of treatment in weeks, also the data contains hematology information such as the white blood cells count (WBC), platelets (PLT), hemoglobin level (HGB), and absolute neutrophil count (ANC) were recorded and written down by the researcher who attended Sundays and Tuesdays in the OP clinic of CWTH which receives the patients during their routing visits for checkup and chemotherapy treatment. Finally, information about the dosage of 6-mercaptopurine (6-MP), in addition to the general clinical condition and the treatment gaps were recorded during checking the patients' own registry book. Those subjects were receiving Vincristine monthly, Methotrexate (MTX) weekly, 6-MP daily, five-day-course of dexamethasone monthly, and Intrathecal chemotherapy (MTX) every three months as per protocol guidelines.

Methods:

This is a cross sectional study in which Thiopurine S-methyltransferase (TPMT) activity was measured for all the patients in the study sample. Two ml of peripheral blood in ethylene diamine tetra acetic acid (EDTA) tube was obtained from each patient and frozen at –20^{o}C till used for the genotyping methods. All blood samples were analyzed to detect genomic DNA, first of all by extracting the DNA from the blood using ExiPrep ™ Plus Blood Genomic DNA Kit (K-4211) provided by Bioneer, Korea, and then amplifying the extracted DNA with predesigned specific primers synthesized by Bioneer, Korea, followed by gel electrophoresis to separate and detect these amplicons. Additionally, all sera were analyzed for Liver Function Tests (LFT) including Alanine Aminotransferase ALT (GPT), Aspartate Aminotransferase AST (GOT), Alkaline Phosphatase (ALP) and Total Serum Bilirubin (TSB) in addition to Complete Blood Count (CBC) which is already requested by the treating physician as part of follow up. The DNA extraction and PCR procedure were carried out in Central Health Lab that belongs to the Ministry of Health and Teaching Lab of Baghdad College of Medicine which belongs to the University of Baghdad during the period from January to April 2018, where the researcher was attending these labs during the day time work.

DNA Extraction: Hundred microliter of Genomic DNA from each sample was extracted. These samples were analyzed by agarose gel electrophoresis to determine if it is suitable for PCR reaction after the extraction and then the isolated DNA samples were kept in safe lock tube prepared specially for DNA samples and stored at -20 °C until use.

Polymerase Chain Reaction (PCR): The PCR reaction was carried out in total volume 25µl, by using AccuPower Gold Multiplex PCR Premix (Bioneer Corporation, Korea), containing 10µl of premix, 5µl DNA and 1µl of each primer (forward and reverse) and 8µl of distill water for total reaction volume completing. PCR assay consisted of pre-denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1min, 2 min at the specified annealing temperature, followed by extension step 1 min at 72°C and the final extension step was 5 min at 72°C. The analyzed PCR amplicons were detected by electrophoresis technique with 2% agarose gel.

Detection of TPMT Wild-Type Gene (TPMT*1) Allele: An allele-specific PCR was used to analyze the TPMT *1 allele, using sequence specific primers: P2FW 5′GTATGA TTT TAT GCA GGT TTG 3′ and P2R 5′TAA ATA GGA ACC ATC GGA CAC 3′ DNA fragment was amplified with P2W and P2C primers by PCR assay with annealing temperature of 53°C for 2 min. When wild-type was present the PCR reaction yielded 245 base pairs detected by electrophoresis with 2% agarose gel. A band at 245 bp (corresponding to TPMT*1 allele) was always present due to heterozygote allele.

Allele-Specific Multiplex-PCR: An allele-specific multiplex-PCR [11] was used to detect the TPMT mutant alleles G460A and A719G (TPMT*3A), G460A (TPMT*3B) and A719G (TPMT*3C) using specific primers for exon 7 and 10 in TPMT gene (Table 1).

Table 1: Primers used to detect mutations in the TPMT gene and product length for PCR

Exon	Name of Primer	Sequence of Primer	Product Length (bp)	Annealing Temperature(C°)
7	460F	5`-GGGACGCTGCTCATCTTCT-3`	338	59
	460R	5`-GCCTTACACCCAGGTCTCTG-3`	338	59
10	719F	5`-AAGTGTTGGGATTACAGGTG-3`	273	56
	719R	5`-TCCTCAAAAACATGTCAGTGTG-3`	273	56

An allele-specific multiplex-PCR is a simple method for detection of more than one single nucleotide polymorphisms (SNPs) in a single PCR reaction by using more than one sequence-specific PCR primer pairs within the single reaction followed by gel electrophoresis only. The produced amplicons should be of different sizes and with melting temperature of at least 2C° differences to be analyzed by one reaction. This technique allows detection of multiple DNA sequences within the same PCR reaction mixture.

Detection of G460A (TPMT*3B) Mutation: Genotype analysis for G460A point mutation at exon 7 was carried out by PCR assay as mentioned above except that the annealing temperature was 59°C for 2 min with primer sequence of 460F and 460R. The fragments yield from the PCR amplifications were 338 base pairs which were detected by electrophoresis technique with the use of agarose gel.

Detection of A719G (TPMT*3C) Mutation: Genotype analysis for A719G point mutation at exon 10 was carried out by PCR assay as mentioned above except that the annealing temperature was 56°C for 2 min with primer sequence of 719F and 719R. The fragments yield from the PCR amplifications were 273 base pairs which were detected by electrophoresis technique with the use of agarose gel.

Detection of G460A and A719G (TPMT*3A) Mutation: Genotype analysis for TPMT*3A allele that carried both the G460A and A719G mutations in the same allele were identified by detecting of G460A and A719G single nucleotide polymorphism (SNPs) in the same sample. The presence of a band at 338 bp was corresponding to TPMT*3B allele, a band at 273 bp was corresponding to TPMT*3C allele and the presence of 338 bp together with 273 bp bands in the same sample is corresponding to TPMT*3A allele.

Statistical Analysis: All statistical analyses were performed using the SPSS for Windows, version 22.0 (SPSS Inc., Chicago, IL). Frequency differences of proportion in TPMT polymorphisms were tested by t- test. The level of significance of p-value < 0.05 was considered statistically significant for all analyses.

Results:

The study included 81 ALL patients during therapy (maintenance phase of treatment according to UKALL 2011 protocol), with 59 boys (72.8%) and 22 girls (27.1%). Three most common inactive alleles of the TPMT gene (TPMT*3A, TPMT*3B and TPMT*3C) were sought. The age ranged from 22 months to 16 years (1.83-16.25 years) with a mean of (7.08 ± 3.12) years. A total of 51 children with allele frequencies of 62.96% were homozygous for the wild-type allele (TPMT*1) which means that they did not carry any of the three detected mutations. Thirty children with allelic frequency of 37.03% were heterozygous for one of the two mutant alleles (TPMT*3A or TPMT*3C), while TPMT*3B allele was not detected in this study sample. Twenty-four children were having TPMT*3A with allele frequencies of 29.62% and 6 children were detected with TPMT*3C with allele frequencies of 7.4% (Table 2 and Figure 1).

Table 2: Genotype frequencies of TPMT variants in a sample of 81 pediatric patients

Allele	SNP Position	rs	Amino acid Substitution	Gender		No. of Allele	Frequency %
				M	F		
TPMT*1	Wild type			37	14	51	62.96
TPMT*3A	G460A A719G	1800460 1142345	Ala 154 Thr Tyr 240 Cyc	18	6	24	29.62
TPMT*3B	G460A	1800460	Ala 154 Thr	0	0	0	0.00
TPMT*3C	A719G	1142345	Tyr 240 Cyc	4	2	6	7.42
Total				59	22	81	100

In the current study, forty-two patients (51.8%) (were labeled as intolerant to therapy for whom the dosing of 6-MP was modified and adjusted to reduce the resultant toxicity (30 Myelosuppression and 12 Jaundice) while 39 patients (48.1%) were receiving their standard doses of 6-MP without apparent toxicities on clinical background. The decision for adjusting the dosing in the intolerant group was depending solely on clinical suspicions (prolonged neutropenia and/or Jaundice). Only 12 patients (11 jaundice and 1 Neutropenia) out of the 42 intolerant patients were homozygote for wild-type (TPMT*1) allele and no defective alleles were recognized in their genotypic analysis. The other 39 tolerant patients showed only 2 patients with defective mutant alleles while all other cases were homozygous for the wild-type.

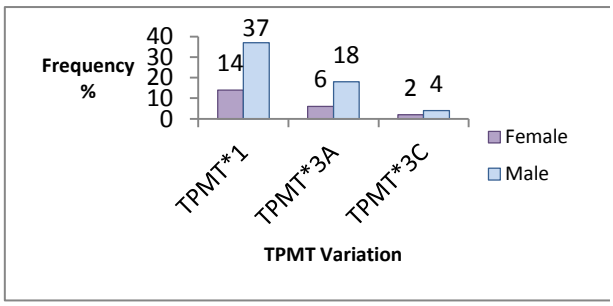

Figure 1: The distribution of TPMT polymorphisms in the study sample

Discussion:

TPMT is the main enzyme participating in thiopurine drugs metabolism, therefore optimal adjustment of drug dose may be useful for optimizing 6-MP therapy to reduce the risk of developing myelosuppression and even death of ALL patients who have low TPMT activity receiving standard dosages of mercaptopurine [13], but such patients can be successfully treated, without severe toxicity, if the dose is properly adjusted [14]. It has been well recognized, that the balance between 6-MP and toxic thioguanine nucleotides (TGNs) in patients treated with thiopurine drug depends on TPMT activity and consequently the genetic variation of this enzyme [15]. In the absence of pre-symptomatic genetic analysis of TPMT polymorphisms, the majority of those patients are at risk of rapidly developing severe myelosuppression due to a mutation in the gene coding for the TPMT enzyme [16]. Knowledge of the molecular basis for low TPMT activity is important for reducing drug-induced toxicity effects, therefore in the present study , three of the most prevalent TPMT mutant alleles TPMT*3A, TPMT*3B, TPMT*3C were genotyped in 81 ALL children as a part of their ALL treatment in which they were receiving mercaptopurine (6-MP) drug. In the present study, the TPMT*3A and TPMT*3C were the only deficient alleles detected in our sample population. In Iraqi population, it was found that TPMT*3A deficiency allele had high frequency than TPMT*3C deficient allele contribution to the overall frequency. These outcomes are similar to Caucasian population in which the TPMT*3A is the most common variant allele as compared with TPMT*3C mutant allele. However in Caucasian populations TPMT*3A comprises 60–89% of all deficiency alleles whereas TPMT*3C accounts for only 5– 15% of mutant alleles [17]. TPMT*3A and TPMT*3C were the only deficiency alleles detected in the Jordanian population with an allele frequency of 0.59% and 0.30% respectively which is similar to present study finding [18]. In Gaza Strip population, they found only TPMT*3A mutant allele with allele frequency of 0.89% [19]. A study of mutant allele in Libyan population reviled the presence of TPMT*3A and TPMT*3C with allele frequencies of 0.61% and 1.02% respectively [20]. Many German Caucasian, French Caucasian, British Caucasian, Swedish, Italian, Bulgarian, Iranian, Turkish, American, African American, Mexican and Brazilian population have TPMT*2, TPMT*3A and TPMT*3C mutant allele [21], whereas in Egyptian, Thai, South-east Asian, Japanese, Chinese and Indian populations their own analyses found only TPMT*3C mutant allele [18]. The present study showed a higher frequency of defective TPMT*3A and TPMT*3C alleles in comparison with other studies mentioned, this is largely due to the sample chosen by the investigator and the supervising physician in which 42 patients (out of the total 81 patients) were labeled as intolerant during the course of the disease either because of persistent neutropenia or Jaundice (with negative other investigations) and dosing of the oral chemotherapy (6MP) was adjusted frequently to render them able to continuously receive their assigned treatment but with a lower dosing. Only 12 patients out of the 42 intolerant patients were homozygote for wild-type (TPMT*1) allele and no defective alleles were recognized in their genotypic analysis. The absence of predominant types of TPMT enzyme mutations and the presence of myelosuppression in these three patients may result from the existence of other mutant alleles of TPMT or mutation in other enzymes involving in 6-MP metabolism or other non-genetic factors. In Conclusion, TPMT*3A and TPMT*3C were the only deficiency alleles detected in the ALL patients. TPMT genotyping can provide an important molecular biomarker for predicting the response of high-risk group to the anticancer therapies and minimize the toxic effects of thiopurine therapy.

Authors' Contribution:

Nawar S Mohammed: The current study is a part of PhD thesis requirements, which was conducted in Biochemistry Department / College of Medicine / University of Baghdad.
Prof. Dr. Manal K Rasheed : Supervisor
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الكشف عن وجود طفرات الانزيم ثايوبيورين مثايل ترانسفيريز
(TPMT*3A و TPMT*3B وTPMT*3C) في الاطفال العراقيين المصابين بسرطان الدم اللمفاوي الحاد

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الخلاصة:
المقدمة : ان سرطان الدم اللمفاوي الحاد هو اكثر الامراض السرطانيه حدوثا في الاطفال، الذي يعالج باستخدام العلاج الكيمياوي فقط. الثايوبيورين هو دواء اساسي في كل الطرق الحديثة المتفق عليها لمعالجة سرطان الدم اللمفاوي، حيث يعمل كعامل مضاد للسرطان ولفتره طويله من العلاج، وقد يؤدي هذا الى التسمم الدوائي عند هؤلاء الاطفال ويعود السبب الى قلة فعالية انزيم الثايوبيورين ميثايل ترانسفيريز والذي هو احد الانزيمات المسؤوله عن العملية الايضيه لدواء الميركابتوبيورين المستخدم في العلاج، ان هذا النقص في الفعاليه الانزيميه يعود بصوره كبيره الى الطفرات الجينيه الحاصله في جين هذا الانزيم.
الهدف: الكشف عن الطفرات الاكثر تكرارا لانزيم ثايوبيورين ميثايل ترانسفيريز في الاطفال العراقيين المصابين بسرطان الدم اللمفاوي الحاد وتحديد النسب المئويه لتكراراتها.
طرق العمل: شملت هذه الدراسة 81 طفل عراقي مصاب بسرطان الدم اللمفاوي الحاد في مرحلة الـ (maintenance) من العلاج الكيمياوي من ضمن البرنامج العلاجي البريطاني (UKALL) لسنة 2011،وبعد اجراء عملية الاستخلاص للحمض النووي من دم هؤلاء الاطفال اجري الكشف عن وجود الطفرات في هذا الانزيم بواسطة تفاعل التضاعف المتسلسل (PCR).
النتائج : اظهرت النتائج ان الانزيم لـ 51 طفل كان طبيعي لايحوي اي طفره جينيه وبتردد تكراري (62.96%) و 30 طفل كان لديهم طفره جينيه في الانزيم وبتردد تكراري (37.03%) اما للاليل (TPMT*3A) بتردد تكراري (29.62%) او للاليل (TPMT*3C) وبتردد تكراري (7.4%) . واما الطفره الجينيه بالاليل (TPMT*3B) فلم تظهر في هذه المجموعه من المرضى. لقد وجدنا في هذه الدراسة الطفرات الجينيه (TPMT*3A) و (TPMT*3C) في جين الانزيم الناقل لمجموعة المثيل (TPMT) ولم نجد الطفره الجينية (TPMT*3B). هذه الدراسة هي الاولى في تحليل الطفرات الجينيه للانزيم ثايوبيورين مثايل ترانسفيريز في عينه من اطفال المجتمع العراقي المصابين بسرطان الدم اللمفاوي اثناء مرحلة العلاج الكيمياوي.
الكلمات المفتاحية: سرطان الدم اللمفاوي، ميركابتوبيورين، ثايوبيورين ميثايل ترانسفيريز، الطفرات الجينية.