

Effects of Thiopurine S-methyltransferase Genetic Polymorphism on Mercaptopurine Therapy in Pediatric ALL

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Abstract: Background: Mercaptopurine is one of the most important drugs used in cancer treatment. Its elimination depends mainly on the enzyme Thiopurine S-methyl Transferase (TPMT). A number of known genetic polymorphisms can affect the activity of this enzyme. **Aim of the work:** to study the pattern of TPMT polymorphisms in a cohort of Egyptian patients with ALL and its impact on response to therapy and toxicity. **Patients and methods:** the study included 52 low-risk pediatric ALL patients treated by PNCI XIII LR Protocol. TPMT genotype was done for common mutations using polymerase chain reaction-based assays. Clinical follow-up, documentation of events, and 6-MP dose reductions were performed throughout the maintenance phase in a double blinded fashion. **Results:** TPMT genotyping showed that 40 patients (76.9%) have the wild type and 12 (23.1%) have the mutant type, two (3.85 %) of which were homozygous for G238C and ten were heterozygous (19.25%). TPMT mutant patients, especially homozygous, were at greater risk of 6-MP toxic effects and needed more frequent dose reductions. Mean duration of missed therapy was 50.250 weeks for the mutant patients vs. 25.825 weeks for wild-type patients (**P < 0.001**). **Conclusion:** TPMT heterozygous and homozygous patients require lower doses of 6-MP. These results justify performing TPMT genotyping before initiating 6-MP therapy in all children with ALL to minimize consequent toxicity through dose modifications.

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Introduction

Antimetabolites are one of the most chemotherapeutic agents used in cancer therapy. They are structural analogues of important cofactors or intermediates in the DNA and RNA biosynthetic pathways. Antimetabolites inhibit synthesis of nucleic acids and their building units or are incorporated into the DNA or RNA, resulting in impaired synthesis. Antimetabolites like methotrexate, mercaptopurine and fluorouracil are folate, purine and pyrimidine analogues respectively. They are used in the treatment of childhood cancers (1).

Mercaptopurine and thioguanine are thiopurines. They are thiol-substituted derivatives of the naturally occurring purine bases hypoxanthine and guanine, respectively. Mercaptopurine has been used in the treatment of ALL for five decades, mainly during maintenance therapy. 6-Mercaptopurine is administered orally at a dose of 75 to 100 mg/m² per day with upward or downward dose adjustments based on the degree of myelosuppression during maintenance therapy. One of the most important factors in the outcome of pediatric ALL is to ensure that patients are

receiving their maximum tolerated dose of mercaptopurine (2). When the actual dose of mercaptopurine received increased by 22% as a result of more aggressive prescribing guidelines, the relapse-free survival improved by 18% (3). High-dose intravenous infusions of mercaptopurine (1,000 mg/m² over 6 to 24 hours) have also been used to overcome the pharmacokinetic limitations of oral dosing (4).

Thiopurines are very useful drugs, but they have a relatively narrow therapeutic index, with life-threatening myelosuppression as a major toxic effect. They are prodrugs that must be converted intracellularly to thioguanine nucleotides to exert a cytotoxic action. These drugs are metabolized, in part, by the cytoplasmic enzyme Thiopurine S-methyltransferase (TPMT) (6).

The level of TPMT activity in human tissues is controlled by a common genetic polymorphism. Individuals who inherit a deficiency in this enzyme exhibit profound intolerance to thiopurine medications, including mercaptopurine and thioguanine (7). TPMT-deficient patients treated with usual doses of these medications, develop profound hematopoietic toxicity

that precludes the administration of other chemotherapy and can be fatal; so reduction of doses by 10-15 folds should be done to prevent the occurrence of such toxicity (8). One in 300 inherit two mutant TPMT alleles and are TPMT deficient, and about 5%-10% are heterozygotes at the TPMT gene locus and have intermediate enzyme activity (2). The rare TPMT-deficient individual probably accounts for most of the thiopurine-intolerant patients who were previously considered to have 'idiosyncratic' toxic effects. TPMT heterozygotes constitute approximately 10% of the patients who receive these medications. If these heterozygotes have intermediate intolerance to thiopurines, due to their intermediate level of TPMT enzyme activity, this would provide a reasonable rationale for routinely assessing TPMT genotype in all patients before starting thiopurine therapy. (7, 9-13). However, another report did not approved these rationale (14)

TPMT exhibits genetic polymorphism in all large ethnic groups studied to date, including Caucasians, Africans, African- Americans, and Asians (15).

Patients and Methods

In this study we studied TPMT polymorphisms in a cohort of 52 newly diagnosed low risk pediatric acute lymphoblastic leukemia (ALL) patients. We aimed to investigate the prevalence of the polymorphic allele and its possible impact on 6-MP tolerability. ALL patients were treated at the NCI, Cairo University, Egypt in the period from June 2005 to October 2008

The study was approved by the IRB of the National Cancer institute, Cairo University. A signed consent from patient's parent had been taken to be enrolled in the study. Diagnosis was performed according to standard clinical, morphological, cytochemical, and immunophenotypic criteria. The recruited patients comprised 36 male and 16 female with an age range of 1-9 years with a median of 5 years.

Inclusion Criteria (Low Risk Criteria)

- Age= 1-9 years (inclusive) at diagnosis
- Total Leukocyte Count (TLC) < 50,000/mm³ at diagnosis
- B-cell progenitor immunophenotype
- No t(1;19), t(9;22), or MLL gene rearrangement by cytogenetics
- CNS status 1 or 2 (WBC < 5/mm³ in CSF)
- Day 15 (mid-induction) bone marrow aspirate (BMA) shows 5% blasts.

Exclusion Criteria:

- Patients with impaired liver or kidney function.

Treatment Protocol

Patients were enrolled into the ALL LR PNCI XIII/2002 Treatment Protocol. In brief, remission induction therapy consisted of prednisone, vincristine,

L-asparaginase, doxorubicin, etoposide, cytarabine, and triple intrathecal injections (methotrexate, hydrocortisone, and cytarabine) given over a 4-week period. Two weeks later, patients started consolidation therapy which was a 14-day phase that consisted of high-dose methotrexate at a dose of 2 g/m² over a 2-hour infusion period given weekly for 2 doses with leucovorin rescue, in addition to 6-mercaptopurine at a dose of 75 mg/m² given daily by the oral route for the whole duration of the phase (14 days). Maintenance phase was then started and continued for 120 weeks. It consisted of weekly methotrexate at a dose of 40 mg/m² given intravenously or intramuscularly, with 6-mercaptopurine at a dose of 75 mg/m² given daily by the oral route for the whole duration of the week. Every 4th week, methotrexate and 6-mercaptopurine were substituted by a pulse of vincristine given intravenously at a dose of 1.5 mg/m² (with a maximum dose of 2 mg/m²) and dexamethasone given orally for the whole week at a dose of 8 mg/m²/day in 3 divided doses. Every 8 weeks during the 1st year only, methotrexate was substituted by high-dose methotrexate like that given in the consolidation phase and a triple intrathecal injection was added. Furthermore, a re-induction window was executed at weeks 16 to 19 and consisted of prednisone, vincristine, L-asparaginase, and doxorubicin. Complete blood counts were obtained weekly. Chemotherapy was given every week, provided that the absolute neutrophil count was 0.3x10⁹/L, platelet count was 50x10⁹/L, and that the patient did not exhibit other complications, such as severe mucositis, infection, or hepatotoxicity.

Study Structure

This study was conducted on a double-blind basis. Once the diagnosis of low-risk ALL was established, blood samples for TPMT genotyping were withdrawn and processed (using serial numbers) without knowing the specific genotype of each patient. Clinical follow-up, documentation of events, and 6-MP dose reductions were performed throughout the maintenance phase, and the resultant data were correlated with the specific TPMT genotypes after ending treatment.

I. Genotyping

Genotyping for TPMT G460A and A719G mutations was performed by PCR-RFLP method (9). TPMT G238C mutation was performed by ARMS (9), in which we used a common primer plus one primer for wild type and another for mutant type in a separate PCR reaction. Absence of these alleles was considered as TPMT wild type.

1. Detection of G238C

Genomic DNA, 400 ng, was amplified under the conditions previously described (9). The final volume for all PCR assays was 25 micro

L. Through use of 1 µg genomic DNA (Qiagen) as a template, PCR was done with primers (table 1) in buffer D (Invitrogen, San Diego, California). The buffer contained Tris hydrochloride (pH 8.5), 60 mmol/L; ammonium sulfate, 15 mmol/L; and magnesium chloride, 3.5 mmol/L. The concentration of each oligonucleotide was 0.1 OU/mL (about 0.5 µmol/L), and 0.2 µL Taq polymerase (Qiagen) was used. With a Biometra T3 thermocycler (Germany), amplification was done for 30 cycles consisting of denaturation at 94 °C for 1 minute, annealing at 55 °C for 2 minutes, and extension at 72 °C for 1 minute. A final extension step at 72 °C for 7 minutes was done. A DNA fragment was amplified with P2M and P2C primers when C238 (mutant) was present, whereas a DNA fragment was amplified with P2W and P2C primers when G238 (wild-type) was present (**Table 1 and Fig 1**).

2. Detection of G460A

PCR was performed in conditions similar to that of G238C except for buffer J (Invitrogen) which contained Tris hydrochloride [pH 9.5], 60 mmol/L; ammonium sulfate, 15 mmol/L; and magnesium chloride, 2.0 mmol/L instead of buffer D (9). MwoI digestion of amplified wild-type DNA yields fragments of 267 and 98 base pairs, whereas DNA containing the G460A mutation is not digested and yields an uncleaved fragment of 365 base pairs (**Table 1 and Fig 2**).

1. Detection of A719G

To detect the A719G mutation, a PCR assay using primers P719R and P719F was performed under the same conditions as those used for the G460A mutation (9), except that we used buffer N (Invitrogen) instead of buffer D. The A719G mutation introduces an AccI restriction site in the amplified fragment and yields fragments of 207 and 86 base pairs. Wild-type DNA yields an uncleaved fragment of 293 base pairs (**Table 1 and Fig 3**).

II-Clinical Follow-up

1. Evaluation of the Toxic Effects of 6-MP

For each weak therapy withheld due to toxicity caused by 6-MP, the main cause was documented (neutropenia or hepatotoxicity) and the week marked as a missed one for treatment. Neutropenia was defined as an ANC (Absolute Neutrophil Count) < 0.3 x 10⁹ cells/L. Episodes of fever and neutropenia necessitating antibiotic treatment - on outpatient or inpatient basis - were also

considered toxic effects of treatment. Hepatotoxicity was defined as 5-fold serum ALT (Alanine Transaminase) elevation or serum direct (conjugated) Bilirubin 2.0 mg/dL in a previously healthy patient not suffering from any of the known hepatitis virus infections, which dropped back to normal values when treatment was withheld.

2. Dose Modification for 6-MP

Patients missing 25% (30 weeks) of treatment, as documented by missed treatment weeks, had 6-MP dose reduced preliminarily by 30% (75 → 50 mg/m²/day). This dose reduction was reviewed every 4 weeks by means of evaluation of the main toxic effects of treatment.

Statistical Analysis

SPSS (Statistical Package for Social Sciences) software version 12.0 was used for data analysis. Mean and standard deviation were estimates of quantitative data. Non-parametric t test compared means of 2 independent groups and ANOVA was used for comparing more than 2 groups with Scheffe Test for post-hoc pairwise comparisons. Chi-Square/Fischer Exact were tests of proportion independence. ROC (Receiver Operator Characteristic) curve analysis was used to set up different levels of neutropenia, hepatotoxicity, and missed treatment durations to categorize 6-MP phenotypes into first, 'Wild' (normal) and 'Mutant' (heterozygous and homozygous) and then, 'Heterozygous Mutant' and 'Homozygous Mutant'. Accordingly, sensitivity and specificity were used to assess the validity of previous measures as diagnostic elements of abnormalities. Significant P value was set at 0.05.

Results

Toxic Effects of 6-MP

The toxic effects of 6-MP among the study group including neutropenia, hepatotoxicity and missed treatment weeks are demonstrated in (table 2).

Dose Reduction for 6-MP

Of the 52 studied patients, 12 (23.1%) required 6-MP dose reduction by 30% (70% of the original dose was given), due to missing 25% (30 weeks) of the duration of maintenance treatment. The other 40 patients (76.9%) required no dose reduction.

TPMT Genotype

The results of TPMT genotyping showed that 40 patients (76.9%) have the wild type (normal, no enzyme defect) and 12 patients (23.1%) have the mutant type. Out of the 12 mutant patients, three (5.77%) showed genotype TPMT *1/TPMT *2 (heterozygous mutant G238C), two (3.85%) showed genotype TPMT *2/TPMT *2 (homozygous mutant G238C), two (3.85%) showed genotype TPMT

*1/TPMT *3A (heterozygous mutant G460A and A719G), four (7.69%) showed genotype TPMT *1/TPMT *3B (heterozygous mutant G460A), and one patient (1.92%) showed genotype TPMT *3B/TPMT *3B (homozygous mutant G460A) (Fig. 4).

Clinical impact of TPMT Polymorphisms

1. Neutropenia

Wild TPMT group (40) showed duration of 13.075 ± 4.0344 weeks vs. duration of 24.5 ± 7.5257 weeks for the mutant group (12) (**p value < 0.001**). Neutropenia duration of 14.5 weeks was a cut-off point between wild and mutant genotypes (Fig. 5).

Comparing heterozygous group versus Homozygous group, the heterozygous group (9) showed duration of 21.667 ± 5.4314 weeks vs. duration of 33.0 ± 7.0 weeks for the homozygous group (3) (**p value = 0.002**). Neutropenia duration of 27.5 weeks was a cut-off point between heterozygous and homozygous genotypes (Fig. 7).

2. Hepatotoxicity

Wild TPMT group (40) showed duration of 8.925 ± 3.5184 weeks vs. duration of 21.250 ± 6.5661 weeks for the mutant group (12) (**p value < 0.001**). Hepatotoxicity duration of 13.0 weeks was a cut-off point between wild and mutant genotypes (Fig. 5).

Comparing heterozygous group versus homozygous group, the heterozygous group (9) showed duration of 18.222 ± 3.0732 weeks vs. duration of 30.333 ± 5.8595 weeks for the homozygous group (3) (**p value < 0.001**). Hepatotoxicity duration of 24.5 weeks was a cut-off point between heterozygous and homozygous genotypes (Fig. 7).

3. Missed Treatment Weeks

Wild TPMT group (40) showed duration of 25.825 ± 6.1306 weeks vs. duration of 50.250 ± 14.5797 weeks for the mutant group (12) (**p value < 0.001**). Missed treatment duration of 31.5 weeks was a cut-off point between wild and mutant genotypes (Fig. 5). Comparing heterozygous group versus Homozygous group, the heterozygous group (9) showed duration of 44.222 ± 8.7433 weeks vs. duration of 68.333 ± 14.4684 weeks for the homozygous group (3) (**p value < 0.001**). Missed treatment duration of 56.0 weeks was a cut-off point between heterozygous and homozygous genotypes (Fig. 7).

On constructing a ROC curve for the toxicities among wild group versus mutant group, area under the curve (AUC) for neutropenia was 0.930 at a cut-off point of 14.5 weeks. For hepatotoxicity, AUC was 0.970 at a cut-off point of 13.0 weeks. AUC for missed treatment

was 0.973 at a cut-off point of 31.5 weeks (Fig. 6). However the ROC curve for the toxicities among heterozygous group versus homozygous group area under the curve (AUC) for neutropenia was 0.926 at a cut-off point of 27.5 weeks. For hepatotoxicity, AUC was 1.000 at a cut-off point of 24.5 weeks. AUC for missed treatment was 0.963 at a cut-off point of 56.0 weeks (Fig. 8)

Discussion

The antimetabolite 6-MP has been used in the treatment of ALL for five decades, primarily for the maintenance of remission. TPMT genetic polymorphism has been investigated intensively over the past decade.

The drug is metabolized by TPMT enzyme; its activity is affected by TPMT gene polymorphisms. Individuals who inherit a deficiency in this enzyme exhibit profound intolerance to 6-MP. There are currently 11 variant alleles associated with low TPMT activity in humans (10). Out of these, TPMT *2, *3A, *3B, and *3C mutations account for more than 95% of the deficiency (11).

Of the 52 patients included in this study, 12 (23.1%) required 6-MP dose reduction by 30% (70% of the original dose was given) due to major toxicities. The other 40 patients (76.9%) required no dose reduction. This correlated perfectly with TPMT genotyping, when it turned out that those 12 patients requiring dose reduction were all TPMT mutants.

In this study TPMT*2 and *3B allele were each encountered in 5 (9.6%) patients. This is much higher than that reported by other studies (table 5). On the contrary TPMT*3C allele was not detected in any of our patients; this is in agreement with some previous studies (9,12,13,14), while most of the other studies reported a higher incidence of TPMT*3C allele (table 3). These differences in overall and specific frequencies could be attributed to ethnic variation or simply to statistical errors resulting from the relatively small sample size used in this study. In this study TPMT*3A was encountered in 2 patients (3.8%) which is comparable to previous reports (9, 12, 14-16).

Clinical Impact of Wild vs. Mutant TPMT

The study proved a very strong clinical impact of TPMT genotype on 6-MP tolerance. The duration of neutropenia, hepatotoxicity and missed treatment weeks were significantly higher in patients harboring the mutant alleles ($p < 0.001$). Duration of 14.5, 13 and 13 weeks was the discriminating cut off for the 3 parameters respectively.

A dose reduction of 30% (70% of the original dose given) was performed on 12 patients due to missing > 25% of the duration of maintenance treatment. Those 12 patients, as we mentioned before, turned out to be all TPMT mutants by genotyping.

In agreement with our results, the cumulative incidence of 6-mercaptopurine dose reductions for myelosuppression was highest among patients homozygous for TPMT deficiency (100% of patients), intermediate among heterozygous patients (35%), and lowest among wild-type patients (7%) ($P < .001$) indicating that *TPMT* polymorphism does have an impact on the optimal dose of 6MP. However hepatotoxicity was more not frequent in those with *TPMT* polymorphisms; in fact, hepatotoxicity even tended to be more frequent among those with higher TPMT activity (P. value .035)(17,18). In agreement with our results Mary et al., (19) reported a significant association between *TPMT* mutant type and the increased missed treatment weeks (P. value= 0.007). Also Pinkel (20) reported that hepatotoxicity was more frequent in TPMT deficient patients. On the contrary, Stanulla et al., (21) reported that hematopoietic toxicity did not differ between heterozygous, homozygous patients or those with wild type for *TPMT*.

In concordance with our results many other studies reported a significant association of 6-MP toxicity with ALL Patients having *TPMT* mutant alleles (2, 10, 22, and 23).

In contrast to our results Desire and his colleagues (24) reported that the presence of *TPMT* polymorphisms did not seem to completely explain the variation in 6-MP toxicity in 66 pediatric patients. They found that three of the 5 patients (60%) heterozygous for *TPMT**2 or *TPMT**3C polymorphisms and 12/61 patients (20%) with wild type *TPMT* genotype had more than 10% of reduction of 6-MP dose ($P = 0.07$).

Our study showed a clear difference in 6-MP tolerance between heterozygous and homozygous mutant

patients with respect to duration of neutropenia, hepatotoxicity and missed treatment duration (P value= 0.002, < 0.001 and < 0.001) respectively.

These results underline the need for marked 6-MP dose reductions (5-10% of the original) in homozygous mutant TPMT patients. These patients have very low levels of TPMT and hence suffer severe toxicities complicating 6-MP administration.

Detection of the four most prevalent *TPMT* mutations (*2, *3A, *3B, and *3C) yielded greater than 95% concordance between *TPMT* genotype and phenotype in Caucasian population (26), and one can anticipate that the molecular diagnosis of TPMT deficiency and heterozygosity will continue to improve as additional mutations are discovered and incorporated into automated high throughput methods (e.g., DNA arrays).

It is important to recognize that when full doses of 6-MP are prescribed to patients with undiagnosed *TPMT* homozygous or heterozygous mutations, this can compromise the ability to deliver all forms of acute lymphoblastic leukemia chemotherapy and thereby jeopardize the chance for cure. However, with the appropriate dose adjustment, these patients can be successfully treated with all components of acute lymphoblastic leukemia therapy including 6-MP (27).

In conclusion this study further emphasized that *TPMT* mutations are significant determinants of tolerance to acute lymphoblastic leukemia chemotherapy that contains 6-MP. Because this genetic polymorphism places at least 10% of the patients at risk for excessive toxicity and failed treatment results, we suggest that *TPMT* genotyping should be performed prior to therapy for all ALL patients to optimize their 6-MP therapy.

Table 1: Detection primer sequence, restriction enzyme, and amplification/digestion product size for each of the TPMT genetic polymorphisms

Genetic Polymorphism	Method of Detection	Primer Sequence	Restriction Enzyme	Size of Product
G238C	PCR-ARMS	P2W (5'-GTATGATTTTATGCAGGTTTG-3') P2M (5'-GTATGATTTTATGCAGGTTTC-3') P2C (5'-TAAATAGGAACCATCGGACAC-3')		
G460A	PCR-RFLP	P460F (5'-ATAACAGAGTGGGGAGGCTGC-3') P460R (5'-CTAGAACCCAGAAAAAGTATAG-3')	MwoI	Mutant: 267 bp + 98 bp Wild: 365 bp
A719G	PCR-RFLP	P719R (5'-TGTTGGGATTACAGGTGTGAGCCAC-3') P719F (5'-CAGGCTTTAGCATAATTTTCAATTCCTC-3')	AccI	Mutant: 207 bp + 86 bp Wild: 293 bp

Table 2: Durations (weeks) of the major toxic effects of 6-MP in the studied 52 low-risk Pediatric ALL cases.

Effect	Neutropenia	Hepatotoxicity	Missed Treatment
TPMT wild	13.075 ± 4.0344	8.925 ± 3.5184	25.825 ± 6.1306
TPMT mutant	24.5 ± 7.5257	21.250 ± 6.5661	50.250 ± 14.5797
Whole group	15.712 ± 6.9489	11.769 ± 6.8014	31.462 ± 13.5117
Range	7-41	3-37	15-85

Table 3: TPMT allele frequency in different countries and populations.

Country	N	Allele Frequency (%)					Reference
		*1	*2	*3A	*3C	Other	
Great Britain	199	94.7	0.5	4.5	0.3	-	(15)
Serbian	100	96	-	3 (3%)	-	1 (1%) *3B	(12)
Germany	1214	91.9	0.25	4.5	0.37	0.2	(16)
Norway							
Saami	194	96.6	-	-	3.1	0.3	(30)
Whites	66	91.7	-	7.6	0.8	-	
Poland	358	96.8	0.4	2.7	0.1	-	(13)
Japan	522	98.4	-	-	1.6	-	(28)
Singapore							
Chinese	271	97	-	0.2	2.8	-	(31)
Malays	217	97.5	-	-	2.3	0.2	
Ghana	217	92.4	-	-	7.6	-	(15)
USA							
Whites	282	94.5	0.5	4.6	0.4	-	(9,30)
Blacks	248	95.4	0.4	0.8	2.4	1	
Russia	995	97.2	0.1	2.3	0.4	-	(14)
Egypt	52	40 (76.9)	5 (9.6%)	2 (3.8%)	0	*3B=5 (9.6%)	Current study

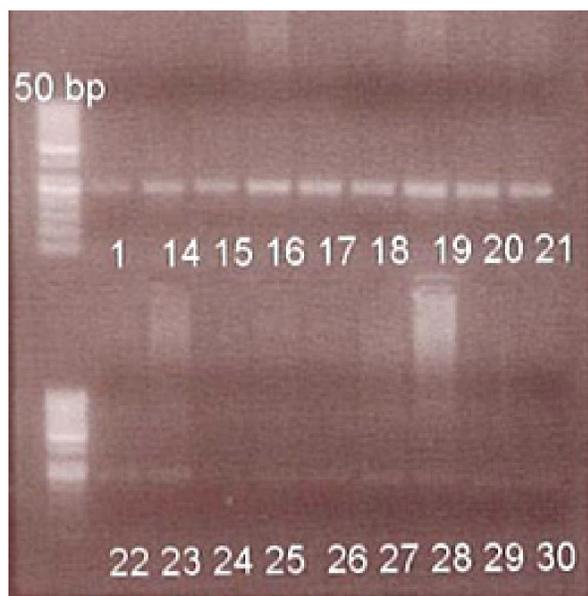


Figure 1 . TPMT G238C Wild Type Amplification

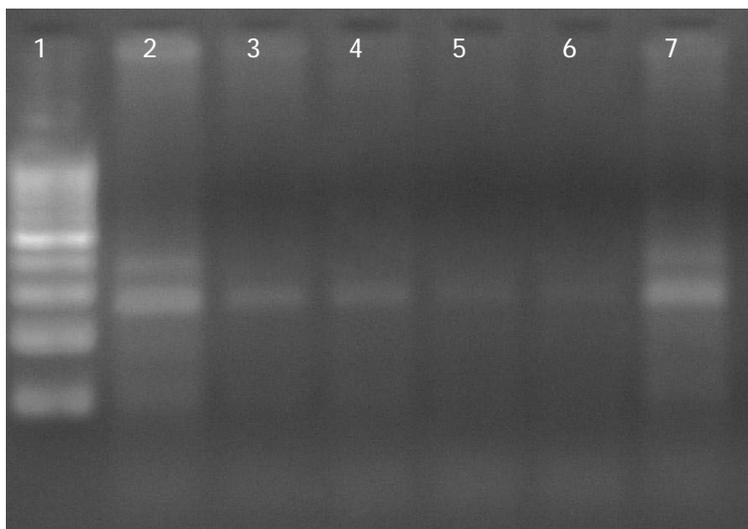


Figure 2. TPMT G460A Genetic Polymorphism.

**Lane 1: 50 base pair ladder,
Lane 2: Heterozygous genotype,
Lanes 3-6: Wild type,
Lane 7: Heterozygous**

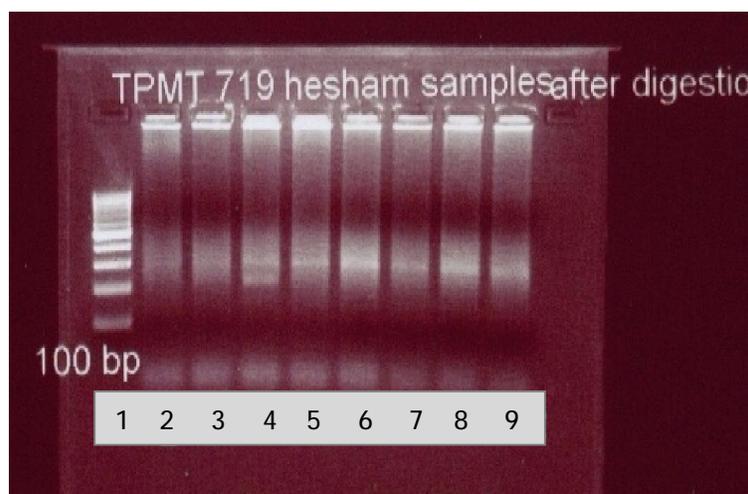


Figure 3. TPMT A719G Genetic Polymorphism.

**Lane1: 100 base pair ladder,
Lanes 2 & 3: Wild type,
Lane 4: Heterozygous genotype,
Lanes 5-9: Wild Type**

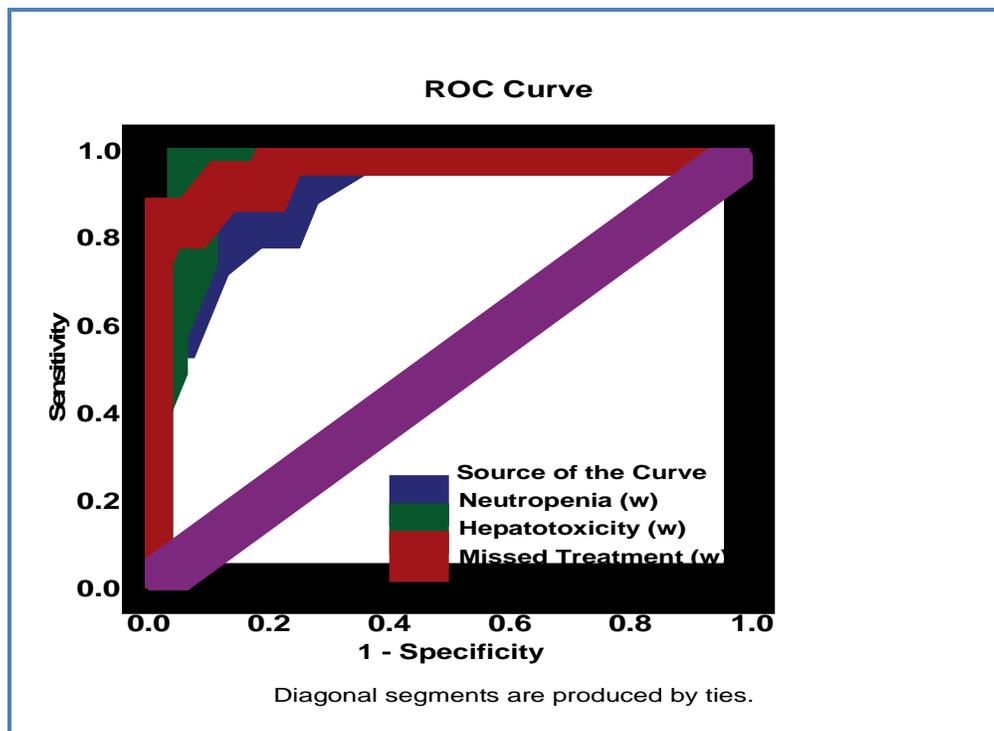


Figure 6. ROC curve discriminating wild from mutant TPMT genotype for neutropenia, hepatotoxicity, and missed treatment durations (weeks) in 52 low-risk Pediatric ALL cases treated with 6-MP.

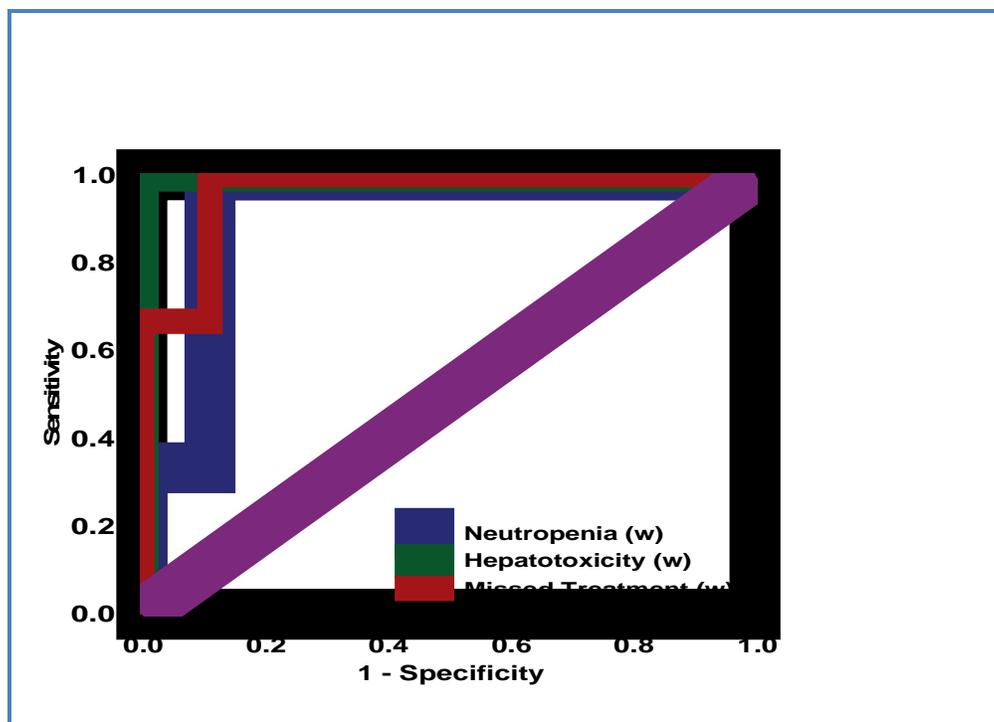


Figure 8. ROC curve discriminating heterozygous from homozygous mutant TPMT genotype for neutropenia, hepatotoxicity, and missed treatment durations (weeks) in 52 low-risk Pediatric ALL cases treated with 6-MP.

Acknowledgements Conflict of interest

Conflict of interest

None of the authors have any conflicts of interest to declare.

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