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Pharmacogenomics

Effect of polymorphisms within methotrexate pathway genes on methotrexate toxicity and plasma levels in adults with hematological malignancies

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Aim: Pharmacogenetics of methotrexate (MTX) contributes to interindividual differences in toxicity. We aimed to evaluate the impact of SNPs within the MTX pathway genes on MTX-induced toxicity and MTX plasma levels at 48 h following treatment in Asian adults with acute lymphoblastic leukemia or non-Hodgkin lymphoma. **Patients & methods:** Patients (n = 71) were genotyped for *MTHFR* C677T, *MTHFR* A1298C, *SLC19A1* G80A, *ABCG2* C421A and *ABCB1* C3435T using the Sequenom MassARRAY® platform. Plasma MTX concentrations at 48 h were measured by fluorescence polarization immunoassay. **Results:** Forty-eight patients had hematopoietic toxicity, 51 had hepatic toxicity and 36 had mucositis. Patients homozygous for *MTHFR* 677TT were associated with increased risk of both hematopoietic (odds ratio [OR]: 9.03; 95% CI: 2.28–36.16; p = 0.002) and hepatic (OR: 3.92; 95% CI: 1.01–15.11; p = 0.036) toxicities. Hepatic toxicity was associated with *SLC19A1* G80A (OR: 5.27, 95% CI: 1.21–22.72; p = 0.032) and *ABCB1* C3435T (OR: 8.62; 95% CI: 1.96–37.57; p = 0.004). However, polymorphisms in *MTHFR* A1298C and *ABCG2* C421A were not associated with any of the toxicities, and mucositis was not associated with any polymorphisms of the MTX pathway genes. Patients with *MTHFR* C677T and *ABCB1* C3435T polymorphisms appear to have significantly higher MTX plasma concentrations (p < 0.05). **Conclusion:** Our results in Asian adults provides evidence for the contribution pharmacogenetics to the toxicity of high-dose MTX and plasma MTX concentrations at 48 h following treatment in patients with acute lymphoblastic leukemia or non-Hodgkin lymphoma. These results will contribute towards the effort of MTX therapy individualization.

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Keywords: high-dose methotrexate • methotrexate plasma concentration • methotrexate toxicity • NHL • non-Hodgkin lymphoma • single nucleotide polymorphism • SNP

Background

The folate inhibitor drug, methotrexate (MTX) has been shown to be beneficial for the treatment of a variety of adult and childhood cancers, including breast cancer [1], osteosarcoma [2], acute lymphoblastic leukemia (ALL) and lymphoma [3]. Intravenous infusion of high-dose MTX (HDMTX; ≥0.5 g/m^2) in combination with other chemotherapeutic agents, as opposed to a conventional dose of <0.5 g/m^2, has been shown to be effective in adult non-Hodgkin lymphoma (NHL) and ALL [4]. However, HDMTX treatment can cause substantial toxicity; leading to nonadherence to treatment, and hence can lead to increased mortality and morbidity [3].

There is considerable interpatient variability in occurrence of toxicity owing to HDMTX, causing unpredictable toxicity even when given in fixed doses to similar cohorts of patients [5]. Toxicity can affect the bone marrow causing myelosuppression, the liver leading to elevated liver enzymes and the

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gastrointestinal tract causing oral mucositis [6]. MTX-induced toxicity is generally owing to the inhibition of normal cells and tissue growth adjacent to the target abnormal cells [7]. In normal clinical practice, plasma MTX monitoring is essential especially for patients who have been administered HDMTX in order to detect those who are at risk for HDMTX-related toxicities and to determine the duration of leucovorin rescue. Identifying genetic predictors of MTX toxicities is important for dosage adjustment and minimization of adverse effects.

MTX action on the MTX pathway involves several metabolizing enzymes and transporters (Figure 1) whose functions have been suggested to be altered by genetic polymorphisms [3]. MTHFR is a key regulator enzyme that is essential for DNA synthesis and DNA methylation [8]. Solute carrier (SLC) transporters are involved in MTX uptake and may modify its toxicity and efficacy. SLC19A1 (also known as RFC1) has a general role in folate transport and mediates the transport of MTX into cells [9]. MTX is pumped out of the cell by a variety of ATP-binding cassette (ABC) efflux transporters [10]. ABCG2 (formerly known as BCRP) and ABCB1 (MDR1) are two ABC family genes. It had been shown that the expression patterns of the *ABCB1* and *ABCG2* genes affect the pharmacokinetics of MTX, which then significantly affects MTX activity and toxicity [6,11]. Increased incidence of toxicity has been well documented in association with polymorphisms in these MTX pathway genes [10–14]. Given these findings, the sheer scale of MTX toxicity might be profoundly affected by genetic polymorphisms in the MTX metabolism pathway.

The bulk of research on MTX to date presents conflicting results, and there are marked differences in pharmacogenetics between various populations [6,15]. While pediatric evidence is abundant, there is limited data regarding adults [16]. Therefore, we investigated the association between polymorphisms in the MTX pathway genes with MTX-associated toxicity in a cohort of adult patients with ALL or NHL treated with HDMTX. In addition, our study provides data on the Asian population, in which there has only previously been one study carried out in adults [17].

Patients & methods

Subjects

The patients included in this study were 71 Malaysian adults with ALL, peripheral T-cell lymphoma (PTCL) and Burkitt's lymphoma. This study was a collaboration between the University Malaya Medical Centre (UMMC) and Ampang Hospital, both of which are located in the city of Kuala Lumpur, Malaysia. The study protocol was approved by the medical ethics committees of both centres. Patients were recruited from hematology clinics between September 2010 and September 2013. The eligible criteria were age ≥18-years-old, not afflicted with other active malignancies and HIV free. A standardized extraction template was used to obtain demographic details, medical history, types of hematological malignancies and laboratory investigations from the medical records. NHL types were classified according to the WHO 2008 classification system [18]. At the time of peripheral blood collection, written informed consent was given by all subjects.

Treatment protocols

The multiagent chemotherapeutic protocols used were the modified German multicentre study group for treatment of adult ALL (GMALL) 07/2003, modified non-Hodgkin Lymphoma–Berlin–Frankfurt–Münster (NHL-BFM) 90 protocol and hyperfractionated cyclophosphamide, vincristine, adriamycin and dexamethasone (hyper-CVAD) protocol-course B [19,20]. All patients received high-dose MTX treatment, particularly 1500 mg/m^2 over 24 h or 1000 mg/m^2 over 24 h for patients diagnosed with ALL, 1500 mg/m^2 over 24 h for Burkitt's lymphoma and 1000 mg/m^2 over 24 h for PTCL patients, followed by leucovorin rescue. The infusion time between the different MTX treatments is the same for the three patient groups.

Toxicity evaluation of MTX

Toxicities were evaluated according to the National Cancer Institute common toxicity criteria (NCI-CTC) Version 2.0 [21] and included hematopoietic toxicity, as well as nonhematopoietic toxicity (hepatic toxicity and mucositis). MTX-induced toxicity was assessed for the period between the administration of MTX and the next course of chemotherapy, whereby the highest grade of toxicity observed in each patient was recorded. The subsequent course of chemotherapy were started during a period of 1–3 weeks after the MTX infusions following resolution of toxicities. Toxicity of any grade was considered as the clinical end point. However, toxicity grade 3 or greater was not measured as an additional end point due to limitation in sample size, which would render the analysis underpowered. Hematologic toxicity was determined by the presence of polymorphnuclear leukocytes $<0.5 \times 10^9$/l, whereas hepatic toxicity was by the presence of an increase in ALP ≥2.5 × upper limit of normal (ULN), and/or bilirubin ≥1.5 × ULN and/or ALT ≥2.5 × ULN and/or AST >2.5 × ULN. Stomatitis was graded according to its severity (grade 1: painless ulcer, erythema or mild soreness in the absence of lesions; grade 2: painful erythema, edema or ulcer, but able to eat or swallow; grade 3: painful erythema, edema or ulcer requiring

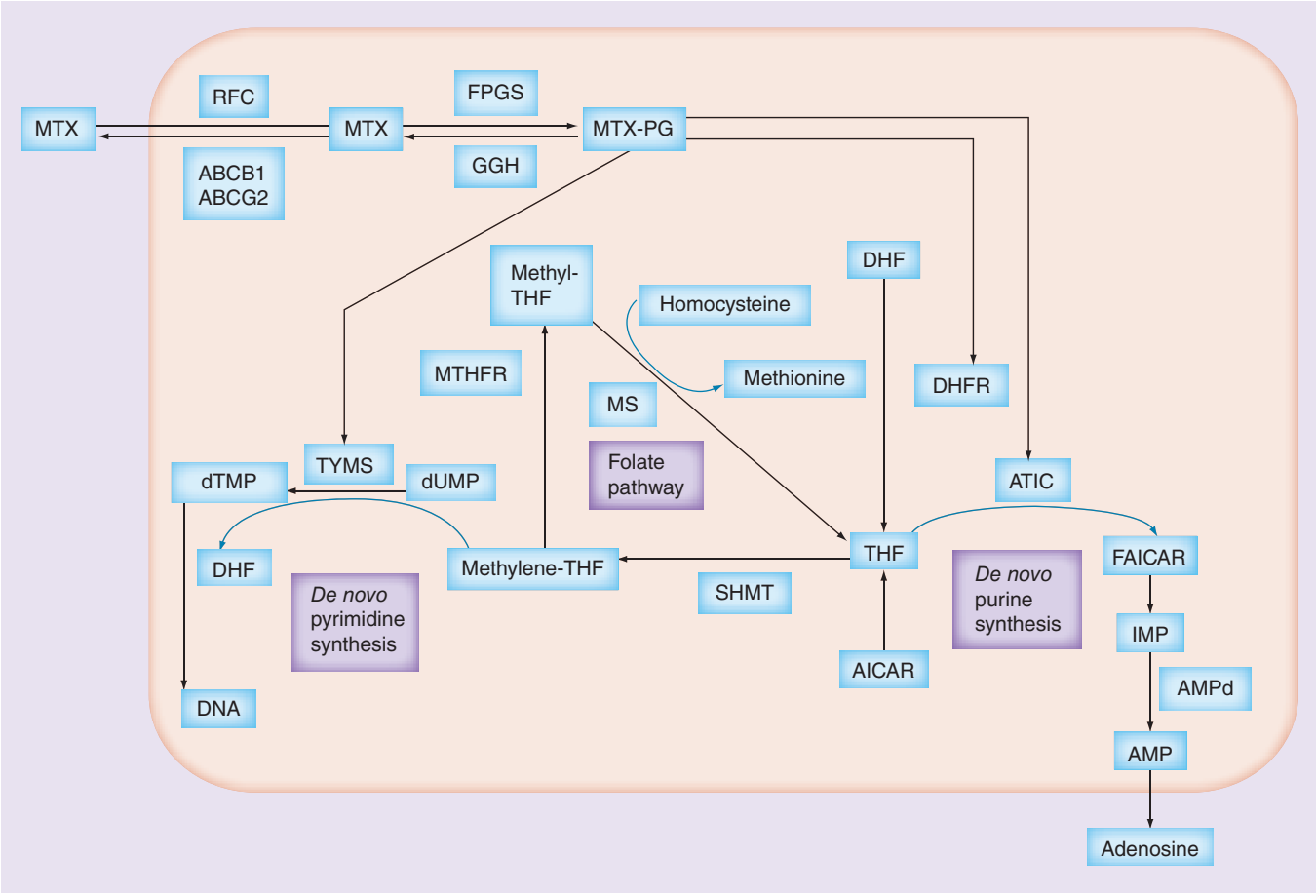

Figure 1. Mechanisms of action of methotrexate. MTX is transported into the cell by RFC1 (SLC19A1). In the cell, MTX is polyglutamated (Glu) by the enzyme FPGS to form MTX-PG. Glutamates can be removed by GGH whereas MTX monoglutamate is removed from the cell through membrane transporters of the ABC family, especially ABCB1 and ABCG2. In the cell, MTX-PGs disrupt the folate metabolic pathway by inhibiting enzymes that are important for DNA synthesis, DNA repair and cell replication. These include DHFR, TYMS and ATIC. DHFR blocks the conversion of DHF to THF, which causes depletion of methionine and decreased DNA methylation. TYMS interferes with *de novo* pyrimidine synthesis. ATIC, which is an enzyme of the *de novo* purine synthesis pathway, causes accumulation of AICAR, which results in increased secretion of adenosine. MTHFR is not directly inhibited by MTX, but is affected by it because of its action in the folate pathway.
ABC: ATP-binding cassette; DHF: Dihydrofolate; FAICAR: 10-formyl 5-aminoimidazole-4-carboxamide ribonucleotide; IMP: Inosine monophosphate; Methyl-THF: 5-methyl-tetrahydrofolate; Methylene-THF: 5,10-methylene-tetrahydrofolate; MTX: Methotrexate; MTX-PG: MTX polyglutamates; THF: Tetrahydrofolate.

intravenous hydration; grade 4: severe ulceration or requires parenteral or enteral nutritional support or prophylactic intubation) [22].

Plasma MTX concentration

The MTX plasma concentrations are consistently examined after HDMTX treatment to determine the rate of drug clearance and the leucovorin dose required for rescue [7]. Various cutoff points based on the MTX plasma half-life have been used to initiate MTX action and to prevent or minimize toxicity. In this study, the MTX plasma concentrations were evaluated at 48 h from the start of the first dose of MTX infusion in accordance with previous reports [23–26]. A few studies have evaluated at both 24 and 48 h [11], and at 48 and 72 h [26], but concluded that significant effect was only seen at 48 h [14,24–26]. Furthermore, assessment of the plasma MTX at 48 h was found to be independent of both treatment protocol and patient's age [23]. Measurement of MTX plasma levels was performed by fluorescence polarization immunoassay according to the manufacturer's instructions (TDx Abbott Laboratories, IL, USA). Leucovorin rescue was started at 42 hafter initiation of MTX infusion at a dose rate of 15 mg/m^2. The effectiveness of leucovorin decreases with time. In addition, MTX toxicity may not be reversible if adequate rescue is delayed for more than 42–48 h. If the MTX level was found to be above the normal MTX level (>1 µmol/l) after 48 h, leucovorin rescue was intensified, and 15 mg/m^2 leucovorin res-

cue dose was given every 6 h until MTX plasma levels reached <0.05 µmol/l [27].

Genetic predictors

Genomic DNA was extracted from the collected blood samples using a QiAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany). The quality of DNA was checked consistently to confirm that 260/280 and 260/230 absorbance ratios exceed 1.8 to indicate high-quality DNA. The genomic DNA was then diluted to 10 and 20 ng/µl, for samples and duplicates, respectively and then placed in the well. A volume of 1 µl of DNA was used in every amplification reaction. The polymorphisms within MTX pathway genes that include *MTHFR* C677T, *MTHFR* A1298C, *SLC19A1* G80A, *ABCG2* C421A and *ABCB1* C3435T were genotyped at the University of Hong Kong, Genome Research Centre using the Sequenom MassARRAY® technology platform with the iPLEX® GOLD chemistry (Sequenom, CA, USA) in the conditions recommended by the manufacturer. MassARRAY AssayDesign software package (v4.0) was used to design the specific assays with proximal SNPs filtering. The quality of the PCR fragment amplification and extension primer specificity was checked prior to running the reaction. Residual nucleotides were dephosphorylated prior to the iPLEX GOLD reaction. Based on a single-base extension, reaction products were desalted with SpectroClean resin (Sequenom), and 10 nl was spotted onto the SpectroCHIP® using the MassARRAY Nanodispenser. The MassARRAY Analyzer Compact MALDI-TOF mass spectrometer was used to determine the mass. The MassARRAY Typer 4.0 software was used for proper data acquisition and analysis. Genotypes were called after cluster analysis using the default setting of the Gaussian mixture model. Inspection of the clusters was carried out to ensure a clear cluster separation with good signal to noise cutoff. A manual review was carried out to further clarify uncertain genotype calls. Assays with less than 80% call rate within the same SpectroCHIP was considered as having failed. A blank and five duplicates were introduced as quality controls. SpectroCHIP with more than 25% call rate in the blank control or less than 99.5% concordance in duplicate checks, and more than 10% call rate in blank checks were considered to have failed and would be required to be repeated.

Statistical analysis

All values were presented as mean ± standard deviation for continuous data and as percentages for categorical data. Deviation from the Hardy–Weinberg equilibrium was assessed using a goodness-of-fit χ^2 where $p < 0.05$ is not consistent with Hardy–Weinberg equilibrium. Logistic regression was employed to examine the association of the MTX pathway gene polymorphisms with MTX-induced toxicity and MTX plasma concentration at 48 h. Multivariate analysis and stepwise regression failed to indicate any contributing factor. Although our initial multivariate analysis did not reveal age and gender as factors, we still included these variables together with different types of diseases in our preanalysis for confirmation, as age and gender are known risk factors associated with adverse events. However, age and gender did not affect the results and there were no significant findings.

Correction for multiple testing was performed using Bonferroni method. All statistical tests were two-sided and results were considered significant if $p < 0.05$. All statistical analyses were performed using the SPSS software version 21.0 (SPSS, IBM Corp., IL, USA).

Results

Patients & distribution of toxicity

Table 1 provides a summary of the characteristics of the patients, their clinical condition and the toxicity experienced. There was no difference in the number of patients developing each kind of toxicity according to the treatment protocol used; thus all patients were analyzed together. The proportion of patients was considered equal between both gender with the median age of all subjects at 34 years. The majority of the patients had ALL (79%) and followed by Burkitt's lymphoma (13%) and PTCL (8%). Hepatic toxicity was the most frequently observed toxicity appearing in 72% of the patients, followed closely by hematopoietic toxicity (68%) and mucositis (51%). Of those with hepatic toxicity, 18 patients (35%) experienced grade 2 or greater. While for hematopoietic toxicity and mucositis, the number documented was 21 (44%) and 10 (28%). There were only four (8%), three (6%) and two (6%) subjects for toxicity grade 3 (hematopoietic, hepatic and mucositis, respectively). However, there are no subjects with toxicity of grade 4 and above.

Pharmacogenetics of MTX

The relationship between SNPs within the MTX pathway genes and therapy-related toxicities among patients is shown in Table 2. All SNP markers studied were in Hardy–Weinberg equilibrium. The *MTHFR* 677TT genotype confers greater risk of hematopoietic toxicity ($p = 0.002$) and hepatic toxicity ($p = 0.036$) when compared with *MTHFR* 677CC. The risk for the 677TT homozygous patients as compared with 677CC is ninefold higher in the former and nearly fourfold higher in the latter. It is also notable that *MTHFR*

677CT genotype is associated with hematopoietic toxicity (odds ratio [OR]: 4.55; 95% CI: 1.15–18.38; p = 0.030). Meanwhile, polymorphism of the *SLC19A1* gene was only found to be associated with hepatic toxicity (OR: 5.27; 95% CI: 1.21–22.72; p = 0.032). The *ABCB1* 3435TT and *ABCB1* 3435TC genotypes were significantly associated with hepatic toxicity (OR: 8.62; 95% CI: 1.96–37.57; p = 0.004; and OR: 4.32; 95% CI: 1.06–17.84; p = 0.040, respectively). Toxicity, however, was not found in *MTHFR* A1298C and *ABCG2* C421A. The p-value remained significant after correction for multiple testing (0.05/5).

Gene polymorphisms & plasma MTX concentration

Assessment of plasma MTX at 48 h was found to be independent of both treatment protocol and patient's age [Suthandiram S *et al.*, Unpublished Data] and was found by Perez *et al.* [23]. We assessed the association between MTX plasma concentration at 48 h with the aforementioned polymorphisms (Table 3). Of all studied SNPs, only *MTHFR* C677T and *ABCB1* C3435T were associated with MTX plasma concentrations ($p < 0.05$). The *MTHFR* C677T polymorphism carriers are associated with higher levels of MTX plasma concentration (≥1 μmol l^{-1}), heterozygous 677CT genotype (OR: 4.11; 95% CI: 0.99–16.99; p = 0.030) and homozygous 677TT genotype (OR: 3.57; 95% CI: 0.98–12.97; p = 0.031), when compared with 677CC genotype. Higher levels of MTX plasma concentration was also displayed by patients who carry *ABCB1* 3435TT genotype (OR: 4.63, 95% CI: 1.09–19.71; p = 0.020). The p-value remained significant after correction of multiple testing (0.05/5).

Discussion

The pharmacogenetics study of MTX in hematological malignancies has been receiving much attention owing to the substantial impact it can have on patients' treatment outcome and associated adverse events due to toxicity development. Moreover, different racial groups have been subjected to variability in response to drugs [6]. Table 4 shows the summary of previous studies of MTX pharmacogenetics in hematological malignancies. Notwithstanding that, reports in Asian adults is limited to one study [17]; a study that had a small sample size (n = 44) and was published in Chinese. In this study, we investigated the association between SNPs in the MTX pathway genes, which includes *MTHFR*, *SLC19A1*, *ABCG2* and *ABCB1*, with risk of MTX toxicity and MTX plasma concentration at 48 h in 71 adult Malaysian patients with ALL or NHL treated with HDMTX. Our results showed that *MTHFR* C677T was strongly associated with MTX-induced toxicity.

It has been previously reported that the *MTHFR* C677T and *MTHFR* A1298C variants are associated with decreased MTHFR enzyme activity and thus, present with MTX-related toxicities [55]. The *MTHFR* pathway gene variant C677T has been found to be associated with increased risk of both hematopoietic and hepatic toxicities in Egyptians [22,28,32], Europeans [5], Asians [17] and in our study. In Spanish [34], Chinese [25] and European [12] subjects, C677T is only associated with increased hematopoietic toxicity, while only increased hepatic toxicity has been observed in mixed populations where the majority are Europeans [31]. In Turkish [39] and European [43] populations, C677T is associated with decreased risk of hematopoietic toxicity, whereas decreased risk of both hematopoietic and hepatic toxicity were observed in another study of Europeans [46]. By contrast, the absence of any association between C677T and MTX toxicities have been shown and summarized in several reviews [6,56]. The

Table 1. Characteristics of patients, their clinical condition and toxicity experienced.

Patients	n (%)
Mean age ± SD (years)	36.6 ± 14.2
Median age, years (range)	34.0 (18–65)
Gender, n (%)	
Male	36 (51)
Female	35 (49)
Disease, n (%)	
ALL	56 (79)
PTCL	6 (8)
BL	9 (13)
Toxicity, n (%)	
Hematopoietic	48 (68)
– Grade I	27 (56)
– Grade II	17 (36)
– Grade III	4 (8)
Hepatic	51 (72)
– Grade I	33 (65)
– Grade II	15 (29)
– Grade III	3 (6)
Mucositis	36 (51)
– Grade I	26 (72)
– Grade II	8 (22)
– Grade III	2 (6)

ALL: Acute lymphoblastic lymphoma; BL: Burkitt lymphoma; PTCL: Peripheral T-cell lymphoma; SD: Standard deviation.

Table 2. The relationship between *MTHFR* C677T, *MTHFR* A1298C, *SLC19A1* G80A, *ABCG2* C421A and *ABCB1* C3435T polymorphisms with therapy-related toxicities (grades 0 vs grade I–IV).

Poly-morphisms (n)	Hematopoietic toxicity				Hepatic toxicity				Mucositis toxicity			
	Grade 0, n (%)	Grade I–IV, n (%)	p-value	OR† (95% CI)	Grade 0, n (%)	Grade I–IV, n (%)	p-value	OR† (95% CI)	Grade 0, n (%)	Grade I–IV, n (%)	p-value	OR† (95% CI)
***MTHFR* C677T**												
CC (15)	10 (44)	5 (10)	–	1	7 (35)	8 (16)	–	1	5 (14)	10 (28)	–	1
CT (23)	7 (30)	16 (33)	0.030‡	4.55 (1.15–18.38)	7 (35)	16 (31)	0.311	2.02 (0.53–57.72)	10 (29)	13 (26)	0.531	0.64 (0.17–12.52)
TT (33)	6 (26)	27 (56)	0.002‡	9.03 (2.28–36.16)	6 (30)	27 (53)	0.036‡	3.92 (1.01–15.11)	20 (57)	13 (36)	0.092	0.33 (0.09–01.17)
***MTHFR* A1298C**												
AA (24)	8 (35)	16 (33)	–	1	8 (40)	16 (31)	–	1	9 (26)	15 (42)	–	1
AC (30)	9 (39)	21 (44)	0.789	1.17 (0.37–33.72)	7 (35)	23 (45)	0.424	1.62 (0.52–55.44)	18 (51)	12 (33)	0.100	0.42 (0.13–11.23)
CC (17)	6 (26)	11 (23)	0.900	0.92 (0.28–23.37)	5 (25)	12 (24)	0.793	1.22 (0.32–34.64)	8 (23)	9 (25)	0.544	0.65 (0.19–12.38)
***SLC19A1* G80A**												
GG (15)	6 (26)	9 (19)	–	1	7 (35)	8 (16)	–	1	8 (23)	7 (19)	–	1
GA (28)	12 (52)	16 (33)	0.861	0.89 (0.28–23.18)	9 (45)	19 (37)	0.351	1.86 (0.50–56.70)	14 (40)	14 (39)	0.842	1.15 (0.35–34.02)
AA (28)	5 (22)	23 (48)	0.120	3.05 (0.78–12.60)	4 (20)	24 (47)	0.032‡	5.27 (1.21–22.72)	13 (37)	15 (42)	0.673	1.33 (0.39–34.64)
***ABCG2* C421A**												
CC (22)	5 (22)	17 (36)	–	1	6 (30)	16 (32)	–	1	11 (31)	11 (31)	–	1
CA (28)	9 (39)	19 (41)	0.461	0.62 (0.18–12.24)	8 (40)	20 (40)	0.921	0.94 (0.28–23.26)	15 (43)	13 (37)	0.803	0.86 (0.25–22.65)
AA (20)	9 (39)	11 (23)	0.130	0.38 (0.12–11.33)	6 (30)	14 (28)	0.853	0.86 (0.27–23.34)	9 (26)	11 (32)	0.753	1.25 (0.36–34.12)
***ABCB1* C3435T**												
CC (13)	5 (22)	8 (17)	–	1	8 (40)	5 (10)	–	1	7 (20)	6 (17)	–	1
TC (26)	10 (43)	16 (33)	1.001	1.00 (0.26–23.94)	7 (35)	19 (37)	0.040‡	4.32 (1.06–17.84)	13 (37)	13 (36)	0.822	1.16 (0.31–34.43)
TT (32)	8 (35)	24 (50)	0.373	1.88 (0.48–47.42)	5 (25)	27 (53)	0.004‡	8.62 (1.96–37.57)	15 (43)	17 (47)	0.675	1.30 (0.36–34.80)

†ORs and 95% CIs were estimated using logistic regression analysis adjusted for diseases.
‡Significant p-value.
OR: Odds ratio.

differences in the findings probably reflect the differences in the genetic pools between various populations studied and the differences in the age group ranging from children (from birth up until 18 years old) [12,17,22,25,31,32,34,39,43,46] to adult [5,28]. More importantly, the absence of a significant contribution of *MTHFR* SNPs to MTX toxicities was only explained by a meta-analysis in the pediatric population [57]. However, two other meta-analyses from the adult studies provided support that the toxicity events are related to polymorphisms in the *MTHFR* gene [15,16]. Our results from Asian adults therefore confirm the adult meta-analyses, thereby affirming that the toxicity events are related to the *MTHFR* C677T polymorphism. This should be taken into consideration in treating patients carrying the TT risk genotypes in order to prevent development of toxicity.

We report the absence of any significant association between *MTHFR* A1298C and MTX toxicities, which is supported by previous studies [10,22,29,35,37,46]. The amino acid change from glutamate to alanine as with the *MTHFR* A1298C mutation causes reduction in enzyme activity leading to more substrate for thymidylate synthesis; this leads to more DNA synthesis and as a result, the risk of toxicity decreases [42]. In addition, the 1298C allele has been found to render a protective effect against various types of MTX toxicities in several reports [25,33,42–44]. Unlike the A1298C variant, decreased enzyme activity in C677T results in increased toxicity risk. *MTHFR* C677T polymorphism is associated with decreased activity of MTHFR and an increased level of homocysteine, which can represent a sensitive and responsive indicator of MTX cytotoxicity between *MTHFR* 677T alleles and *MTHFR* 1298C alleles [5].

The influence of the SLC and ABC families as transporters of MTX across the cell membrane on the folate and MTX pathway genes requires further attention. Studies of genetic polymorphisms in *SLC19A1* G80A and *ABCB1* C3435T in our population showed association with hepatic toxicity. Although several *SLC19A1* G80A reports [41,42,52] failed to support this

Table 3. The relationship between *MTHFR* C677T, *MTHFR* A1298C, *SLC19A1* G80A, *ABCG2* C421A and *ABCB1* C3435T polymorphisms with methotrexate plasma concentration at 48 h.

Polymorphisms	MTX plasma concentration at 48 h			
	≤1 μmol l^{-1}, n (%)	>1 μmol l^{-1}, n (%)	p-value	OR† (95% CI)
***MTHFR* C677T**				
CC	8 (38)	7 (14)	–	1
CT	7 (33)	18 (36)	0.030‡	4.11 (0.99–16.99)
TT	6 (29)	25 (50)	0.031‡	3.57 (0.98–12.97)
***MTHFR* A1298C**				
AA	8 (38)	16 (32)	–	1
AC	8 (38)	22 (44)	0.601	1.38 (0.43–44.44)
CC	5 (24)	12 (24)	0.792	1.20 (0.31–34.61)
***SLC19A1* G80A**				
GG	5 (24)	10 (20)	–	1
GA	11 (52)	17 (34)	0.701	0.77 (0.21–22.88)
AA	5 (24)	23 (46)	0.261	2.30 (0.54–59.76)
***ABCG2* C421A**				
CC	6 (29)	16 (33)	–	1
CA	8 (38)	20 (41)	0.922	0.94 (0.27–23.26)
AA	7 (33)	13 (26)	0.591	0.70 (0.19–12.59)
***ABCB1* C3435T**				
CC	6 (28)	7 (14)	–	1
TC	10 (48)	16 (32)	0.652	1.37 (0.36–35.27)
TT	5 (24)	27 (54)	0.020‡	4.63 (1.09–19.71)

†ORs and 95% CIs were estimated using logistic regression analysis.
‡Significant p-value.
MTX: Methotrexate; OR: Odds ratio.

finding while only one study was consistent with the finding [14], caution should be exercised given that these reports were from pediatric studies. More studies, especially from adults, should be carried out in order to agree or refute this finding. On the other hand, there was one published report to date that found no association between *ABCB1* C3435T and MTX-related toxicity in patients with NHL [11]. Although *ABCB1* C3435T has a major role in transcellular transport of anthracyclines (i.e., doxorubicin), these drugs were not administered on the same day as MTX and would therefore be unlikely to interfere with MTX toxicity. As for *ABCG2* C421A, we found no relationship with MTX-related toxicity. Our results are consistent with the results seen in Japanese subjects with ALL or NHL treated with HDMTX [14].

The present study identified no significant influence of *MTHFR* A1298C, *SLC19A1* G80A and *ABCG2* C421A SNPs on MTX plasma levels at 48 h in patients with ALL and NHL. This observation supports findings from the previous report by Shimasaki and colleagues [41]. Our results are also in agreement with a few reports that demonstrated no significant association with MTX plasma levels for *SLC19A1* G80A in a Japanese [26] and a European population [42,50], and *ABCG2* C421A SNP in a Spanish population [58], but contradict the higher MTX plasma levels found for *SLC19A1* 80AA in another European population [54]. However, we showed association between *MTHFR* 677CT and *MTHFR* 677TT with higher MTX plasma levels at 48 h. The MTX plasma level at 48 h has been found to be more prominent in those with the 677TT genotype in the Japanese population [14], which is similar to our findings. Notably, further leucovorin rescue is usually administered when the MTX plasma concentration is higher than 1 μmol l^{-1} [14] due to MTX elimination at the terminal stage that denotes MTX toxicity [59]. Our data suggest that *MTHFR* genotypes may allow us to adopt a precautionary treatment and predict the requirement for additional leucovorin rescue in order to prevent toxicity events. In this study, we also showed *ABCB1* 3435TT genotype to be associated with higher MTX plasma levels at 48 h; however, this is in contrast to the previous study, which found no significant difference between A*BCB1* 3435TT genotype with MTX plasma levels [11].

Our results showed no significant association of chemotherapy-induced mucositis with any polymorphisms in the MTX pathway genes. This lack of association found in our study is in agreement with results reported in published studies for *MTHFR* C677T in Asian [17,25], Egyptian [60] and European [36,45] populations. Our results are also in agreement with a report that demonstrated no significant association between chemotherapy-induced mucositis and the *MTHFR* A1298C, *SLC19A1* G80A and *ABCB1* C3435T SNPs in the European population [33]. Conversely, there was a published report regarding *MTHFR* C677T in the African [22] and European [33] populations and *ABCB1* C3435T in the Turkish population [61] that failed to support this finding. Oral mucositis is a common, possibly severe, adverse effect caused by chemotherapy for hematological malignancies. Reactive oxygen species (ROS) production is the initial phase of chemotherapy-induced mucositis. Mucositis formation is linked with elevated ROS levels [62]. Although we did not measure ROS levels, lack of association between polymorphisms of the MTX pathway genes and mucositis in our study has suggested that ROS levels might not be regulated. Studies have shown that polymorphisms of the MTX pathway genes caused an increase in ROS levels thereby inducing mucositis [61]. The limitation in the study sample size is readily acknowledged, which is usually the case with this type of study design and this limitation has been shared by most of the studies. Almost two-thirds of the studies have a sample size of less than 100 [6,15]. Most of the current studies have adopted the approach of reporting selected types of toxicity while trying not to lose the sample size. Conversely, the limited and heterogeneous sample population in our study may contribute to false-positive results. In addition, the relatively low incidence of the disease in our study population has made sampling more difficult. Although MTX is well acknowledged to result in hematopoietic, hepatic and mucositis toxicities, other chemotherapeutic agents may also contribute to similar adverse events. It appears that while other chemotherapeutic agents may contribute to the toxicity mentioned, they generally have a limited effect on mucositis as compared with MTX. MTX may be secreted in the saliva, leading to increased direct mucotoxicity [63]. As an example, anthracyclines largely cause cardiotoxicity [64] and vincristine can cause neuropathy [65]. Homocysteine and folate levels have been shown to affect the toxicity by regulating the MTHFR enzyme levels [7]. Although we did not address the homocysteine and folate status in this study, it is possible that homozygosity of *MTHFR* C677T will decrease the enzyme activity thereby causing decreased folate and increased homocysteine concentrations, conditions favoring MTX toxicity [10]. Nevertheless, it must be emphasized that the influence of MTX pathway gene variants on MTX toxicity should be accompanied by the pharmacokinetic findings, which was not assessed in our study as was the case with most of the reported studies. Moreover, the relationship between

Table 4. Summary of previous studies of methotrexate pharmacogenetics in hematological malignancies.

Author (year)	Race	Patients (n)	Population	SNP studied	Disease	MTX dose	Toxicity	Ref.
Eissa *et al.* (2013)	Egyptian	50	Adult	*MTHFR* C677T	ALL	Low	Increased risk of hematologic†, hepatic† and gastrointestinal† toxicity	[28]
Erculj *et al.* (2012)	European	167	Pediatric	*MTHFR* C677T	ALL	High	ND	[29]
Chiusolo *et al.* (2012)	European	54	Pediatric, adult	*MTHFR* C677T	ALL, BL, PCNSL	High	ND	[30]
Sepe *et al.* (2012)	Various	557	Pediatric	*MTHFR* C677T	ALL	High	Increased hepatic† toxicity	[31]
El-Khodary *et al.* (2011)	Egyptian	40	Pediatric	*MTHFR* C677T	ALL	High	Increased hematologic†, hepatic† and other toxicity	[32]
Faganel Kotnik *et al.* (2011)	European	64	Pediatric	*MTHFR* C677T	ALL, ML	High	Increased mucositis†	[33]
Salazar *et al.* (2011)	Spanish	141	Pediatric	*MTHFR* C677T	ALL	High	Increased hematologic† and other toxicity	[34]
D'Angelo *et al.* (2011)	European	151	Pediatric	*MTHFR* C677T	ALL	High	Increased other toxicity	[35]
Liu *et al.* (2011)	Chinese	181	Pediatric	*MTHFR* C677T	ALL	High	Increased hematologic† toxicity	[25]
Karathanasis *et al.* (2011)	Cretan	35	Pediatric	*MTHFR* C677T	ALL	High	ND	[36]
Lopez-Lopez *et al.* (2011)	Spanish	115	Pediatric	*MTHFR* C677T	ALL	High	ND	[37]
Tantawy *et al.* (2010)	Egyptian	40	Pediatric	*MTHFR* C677T	ALL	High	Increased hematologic†, hepatic†, mucositis† and other toxicity	[22]
Horinouchi *et al.* (2010)	Japanese	24	Pediatric	*MTHFR* C677T	ALL, LBL	Low	ND	[38]
Ongaro *et al.* (2009)	European	90	Adult	*MTHFR* C677T	ALL	Low	Increased hematologic†, hepatic† and gastrointestinal† toxicity	[5]
Kantar *et al.* (2009)	Turkish	37	Pediatric	*MTHFR* C677T	ALL, NHL	High	Decreased hematologic† toxicity	[39]
Krull *et al.* (2008)	European	48	Pediatric	*MTHFR* C677T	ALL	Patients were treated with various pediatric oncology protocols	Attention-deficit/hyperactivty disorder, neurotoxicity	[40]

†Toxicities as measurements of end points.
ALL: Acute lymphoblastic leukemia; APL: Acute promyelocytic leukemia; BL: Burkitt's lymphoma; High: High-dose methotrexate (≥0.5 g/m²); Low: Low-dose MTX (<0.5 g/m²); LBL: Lymphoblastic lymphoma; ML: Malignant lymphoma; MTX: Methotrexate; ND: No significant difference; NHL: Non-Hodgkin's lymphoma; PCNSL: Primary CNS lymphoma.

Table 4. Summary of previous studies of methotrexate pharmacogenetics in hematological malignancies (cont.).

Author (year)	Race	Patients (n)	Population	SNP studied	Disease	MTX dose	Toxicity	Ref.
Shimasaki *et al.* (2008)	Japanese	20	Pediatric	*MTHFR* C677T	ALL, NHL	Low	Increased other toxicity	[41]
Huang *et al.* (2008)	European	81	Pediatric	*MTHFR* C677T	ALL	High	ND	[42]
Van Kooten *et al.* (2008)	European	88	Pediatric	*MTHFR* C677T	ALL	High	Decreased risk of hematologic† toxicity	[43]
Liu *et al.* (2008)	Asian		Pediatric	*MTHFR* C677T	ALL	High	Increased risk of hematologic†, hepatic† and gastrointestinal† toxicity	[17]
Pakakasama *et al.* (2007)	Thai	76	Pediatric	*MTHFR* C677T	ALL	High	ND	[44]
Imanishi *et al.* (2007)	Japanese	26	Pediatric	*MTHFR* C677T	ALL, NHL	High	ND	[14]
Kishi *et al.* (2007)	North American	240	Pediatric	*MTHFR* C677T	ALL	High	ND	[10]
Ruiz-Arguelles *et al.* (2007)	European	28	Adult, pediatric	*MTHFR* C677T	ALL	High	Increased oral mucositis†	[45]
Chiusolo *et al.* (2007)	European	82	Adult, pediatric	*MTHFR* C677T	ALL	Low	Increased risk of hematologic† toxicity	[12]
Costea *et al.* (2006)	European	186	Pediatric	*MTHFR* C677T	ALL	Low	Decreased risk of hematologic† and hepatic† toxicity	[46]
Shimasaki *et al.* (2006)	Japanese	15	Pediatric	*MTHFR* C677T	ALL, LBL	High	ND	[26]
Chatzidakis *et al.* (2006)	Greek	46	Pediatric	*MTHFR* C677T	ALL	High	ND	[47]
Seidemann *et al.* (2006)	European	484	Pediatric	*MTHFR* C677T	NHL	High	ND	[48]
Aplenc *et al.* (2005)	Mixed	520	Pediatric	*MTHFR* C677T	ALL	High	ND	[49]
Rocha *et al.* (2005)		246	Pediatric	*MTHFR* C677T	ALL	High	ND	[50]
Krajinovic *et al.* (2004)	French-Canadian	201	Pediatric	*MTHFR* C677T	ALL	Low	ND	[51]
Kishi *et al.* (2003)	Mixed (white and non-white)	53	Pediatric	*MTHFR* C677T	ALL	High	ND in neurotoxicity	[52]

†Toxicities as measurements of end points.
ALL: Acute lymphoblastic leukemia; APL: Acute promyelocytic leukemia; BL: Burkitt's lymphoma; High: High-dose methotrexate (≥0.5 g/m^2); Low: Low-dose MTX (<0.5 g/m^2); LBL: Lymphoblastic lymphoma; ML: Malignant lymphoma; MTX: Methotrexate; ND: No significant difference; NHL: Non-Hodgkin's lymphoma; PCNSL: Primary CNS lymphoma.

Table 4. Summary of previous studies of methotrexate pharmacogenetics in hematological malignancies (cont.).

Author (year)	Race	Patients (n)	Population	SNP studied	Disease	MTX dose	Toxicity	Ref.
Chiusolo *et al.* (2002)	European	61	Pediatric, adult	*MTHFR* C677T	ALL, APL	Low	Increased risk of hematologic† toxicity	[53]
Eissa *et al.* (2013)	Egyptian	50	Adult	*MTHFR* A1298C	ALL	Low	Increased risk of hematologic† and hepatic† toxicity	[28]
Erculj *et al.* (2012)	European	167	Pediatric	*MTHFR* A1298C	ALL	High	ND	[29]
Chiusolo *et al.* (2012)	European	54	Pediatric, adult	*MTHFR* A1298C	ALL, BL, PCNSL	High	ND	[30]
Faganel Kotnik *et al.* (2011)	European	64	Pediatric	*MTHFR* A1298C	ALL, ML	High	Decreased risk of hematologic† toxicity	[33]
Salazar *et al.* (2011)	Spanish	141	Pediatric	*MTHFR* A1298C	ALL	High	Increased risk of hematologic† and other toxicity	[34]
D'Angelo *et al.* (2011)	European	151	Pediatric	*MTHFR* A1298C	ALL	High	ND	[35]
Liu *et al.* (2011)	Chinese	181	Pediatric	*MTHFR* A1298C	ALL	High	Increased risk of hematologic†, hepatic†, mucositis† and other toxicity	[25]
Karathanasis *et al.* (2011)	Cretan	35	Pediatric	*MTHFR* A1298C	ALL	High	Increased risk of hepatic† toxicity	[36]
Lopez-Lopez *et al.* (2011)	Spanish	115	Pediatric	*MTHFR* A1298C	ALL	High	ND	[37]
Tantawy *et al.* (2010)	Egyptian	40	Pediatric	*MTHFR* A1298C	ALL	High	ND	[22]
Ongaro *et al.* (2009)	European	90	Adult	*MTHFR* A1298C	ALL	Low	Increased risk of hematologic†, hepatic† and gastrointestinal† toxicity	[5]
Krull *et al.* (2008)	European	48	Pediatric	*MTHFR* A1298C	ALL	Patients were treated with various pediatric oncology protocols	Attention-deficit/hyperactivity disorder, neurotoxicity	[40]
Huang *et al.* (2008)	European	81	Pediatric	*MTHFR* A1298C	ALL	High	Decreased risk of hematologic† and other toxicity	[42]
Van Kooten *et al.* (2008)	European	88	Pediatric	*MTHFR* A1298C	ALL	High	Decreased risk of hematologic† toxicity	[43]
Costea *et al.* (2006)	European	186	Pediatric	*MTHFR* A1298C	ALL	Low	ND	[46]

†Toxicities as measurements of end points.
ALL: Acute lymphoblastic leukemia; APL: Acute promyelocytic leukemia; BL: Burkitt's lymphoma; High: High-dose methotrexate (≥0.5 g/m^2); Low: Low-dose MTX (<0.5 g/m^2); LBL: Lymphoblastic lymphoma; ML: Malignant lymphoma; MTX: Methotrexate; ND: No significant difference; NHL: Non-Hodgkin's lymphoma; PCNSL: Primary CNS lymphoma.

Table 4. Summary of previous studies of methotrexate pharmacogenetics in hematological malignancies (cont.).

Author (year)	Race	Patients (n)	Population	SNP studied	Disease	MTX dose	Toxicity	Ref.
Liu *et al.* (2008)	Asian		Pediatric	*MTHFR* A1298C	ALL	High	Decreased risk of other toxicity	[17]
Pakakasama *et al.* (2007)	Thai	76	Pediatric	*MTHFR* A1298C	ALL	High	Decreased risk of hematologic† toxicity	[44]
Kishi *et al.* (2007)	North American	240	Pediatric	*MTHFR* A1298C	ALL	High	ND	[10]
Chiusolo *et al.* (2007)	European	82	Adult, pediatric	*MTHFR* A1298C	ALL	Low	ND	[12]
Aplenc *et al.* (2005)	Mixed	520	Pediatric	*MTHFR* A1298C	ALL	Low	ND	[49]
Krajinovic *et al.* (2004)	French-Canadian	201	Pediatric	*MTHFR* A1298C	ALL	Low	ND	[51]
Chiusolo *et al.* (2012)	European	54	Pediatric, adult	*SLC19A1* G80A	ALL, BL, PCNSL	High	ND	[30]
Huang *et al.* (2008)	European	81	Pediatric	*SLC19A1* G80A	ALL	High	ND	[42]
Shimasaki *et al.* (2008)	Japanese	20	Pediatric	*SLC19A1* G80A	ALL	Low	ND	[41]
Imanishi *et al.* (2007)	Japanese	26	Pediatric	*SLC19A1* G80A	ALL, NHL	High	Increased risk of hepatic† toxicity	[14]
Kishi *et al.* (2007)	North American	240	Pediatric	*SLC19A1* G80A	ALL	High	Increased risk of gastrointestinal† toxicity	[10]
Shimasaki *et al.* (2006)	Japanese	15	Pediatric	*SLC19A1* G80A	ALL, NHL	High	More treatment interruption, vomiting	[26]
Rocha *et al.* (2005)	European	204	Pediatric	*SLC19A1* G80A	ALL	High	ND	[50]
Kishi *et al.* (2003)	Mixed (white and non-white)	53	Pediatric	*SLC19A1* G80A	ALL	High	ND	[52]
Laverdiere *et al.* (2002)	European	204	Pediatric	*SLC19A1* G80A	ALL	High	ND	[54]
Imanishi *et al.* (2007)	Japanese	26	Pediatric	*ABCG2* C421A	ALL, NHL	High	ND	[14]
Avivi *et al.* (2014)	Israeli	69	Adult	*ABCB1* C3435T	NHL	High	ND	[11]

†Toxicities as measurements of end points.
ALL: Acute lymphoblastic leukemia; APL: Acute promyelocytic leukemia; BL: Burkitt's lymphoma; High: High-dose methotrexate (≥0.5 g/m^2); Low: Low-dose MTX (<0.5 g/m^2); LBL: Lymphoblastic lymphoma; ML: Malignant lymphoma; MTX: Methotrexate; ND: No significant difference; NHL: Non-Hodgkin's lymphoma; PCNSL: Primary CNS lymphoma.

the *MTHFR* C677T polymorphism and MTX toxicity can be described by disturbances in folate levels and by prolonged MTX exposure due to delayed MTX clearance. Most studies to date are limited to only gene effect and disregard the environmental effects of folate and homocysteine. Thus, the actual measure of effect size of the genetic polymorphisms is limited to the fact that these studies vary in terms of the underlying disease, age group, dosage of MTX, folate status, diet, concurrent medications and even toxicity threshold setting. A multicenter study with a larger sample size that combines and standardizes the study setting would be a better approach in the future in order to generate a consistent and definitive result.

In conclusion, this is the first study to identify a significant role of polymorphisms in the MTX pathway genes on MTX toxicity and MTX plasma concentration at 48 h in Asian adults with hematological malignancies. Furthermore, the study confirmed the influence of *MTHFR* C677T on MTX-related toxicities and plasma levels. Our study suggests that pharmacogenetics modifies MTX-related toxicity and plasma levels in adult ALL or NHL patients. Such results could enable tailored therapy based on a pharmacogenetics approach in order to prevent toxicity events. In addition, polymorphisms of *SLC19A1* G80A and *ABCB1* C3435T need to be further investigated as data has suggested that they may have significant roles in MTX toxicity and plasma MTX levels. Genotyping prior to treatment in adult ALL or NHL is likely to be valuable with the aim of adapting MTX therapy and thus reducing MTX-induced toxicities.

Conclusion

In conclusion, this is the first study to identify a significant role of polymorphisms in the MTX pathway genes on MTX toxicity and MTX plasma concentration at 48 h in Asian adults with hematological malignancies. Furthermore, the study confirmed the influence of *MTHFR* C677T on MTX-related toxicities and plasma levels. Our study suggests that pharmacogenetics modify MTX-related toxicity and plasma levels in adult ALL or NHL. Such results could enable tailored therapy based on a pharmacogenetics approach in order to prevent toxicity events. In addition, polymorphisms of the *SLC19A1* G80A and *ABCB1* C3435T polymorphisms need to be further investigated as data has suggested that they have significant roles in MTX toxicity and plasma MTX levels. Genotyping prior to treatment in adult ALL or NHL, is likely to be valuable with the aim of adapting MTX therapy and thus reducing the MTX-induced toxicities.

Future perspective

Our findings may be significant for the further development of treatment strategy in adult ALL or NHL. Our data could help clinicians to determine individuals at greater risk for MTX-associated toxicity. Clinicians might therefore be able advise patients about the possibility of experiencing MTX-related toxicities.

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Executive summary

Background

- High-dose methotrexate (HDMTX) has been shown to be useful in acute lymphoblastic leukemia (ALL) or non-Hodgkin lymphoma (NHL); however, it causes toxicity in the bone marrow, liver and gastrointestinal tract.
- Pharmacogenetics of methotrexate (MTX) and its relationship with plasma concentrations are conflicting, and there is only one study in Asian adults.

Patients & methods

- In our study we investigated the association between polymorphisms in the MTX pathway genes with MTX-associated toxicity in a cohort of adult patients with ALL or NHL treated with HDMTX.

Results

- Hepatic toxicity is significantly higher in patients with *MTHFR* C677T, *SLC19A1* G80A and *ABCB1* C3435T.
- *MTHFR* C677T is also associated with risk of hematopoietic toxicity.
- Higher levels of plasma MTX were found in those with *MTHFR* C677T and *ABCB1* C3435T.

Discussion

- Our results in Asian adults provide evidence for the contribution of pharmacogenetics to the toxicity of HDMTX and plasma MTX concentrations at 48 h following treatment in patients with ALL or NHL.

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No writing assistance was utilized in the production of this manuscript.

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• of interest; •• of considerable interest

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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