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ORIGINAL ARTICLE

Methotrexate consolidation treatment according to pharmacogenetics of *MTHFR* ameliorates event-free survival in childhood acute lymphoblastic leukaemia

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Recent advances in treatment for childhood acute lymphoblastic leukaemia (ALL) have significantly increased outcome. High-dose methotrexate (MTX) is the most commonly used regimen during the consolidation period, but the optimal dose remains to be defined. We investigated the usefulness of the *MTHFR* genotype to increase the MTX dosage in the consolidation phase in 141 childhood ALL patients enrolled in the ALL/SHOP-2005 protocol. We also investigated the pharmacogenetic role of polymorphisms in genes involved in MTX metabolism on therapy-related toxicity and survival. Patients with a favourable *MTHFR* genotype (normal enzymatic activity) treated with MTX doses of 5 g m^{-2} had a significantly lower risk of suffering an event than patients with an unfavourable *MTHFR* genotype (reduced enzymatic activity) that were treated with the classical MTX dose of 3 g m^{-2} ($P = 0.012$). Our results indicate that analysis of the *MTHFR* genotype is a useful tool to optimise MTX therapy in childhood patients with ALL.
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Introduction

Acute lymphoblastic leukaemia (ALL) is the most common paediatric cancer, accounting for 25% of all cancers in children.[1] In the last three decades, great progress has been made in diagnosis and outcome, with 5-year event-free survival (EFS) rates of 70–80% and overall cure rates of 80%.[2] Such an improvement in the treatment outcome is largely due to the optimal use of antileukemic agents and the adaptation of treatment to the clinical risk. Nevertheless, 5% of children do not achieve remission and 10–20% of children either do not respond to treatment or relapse.[3] Besides, interruption of treatment due to toxicity may further increase the risk of relapse.

High-dose methotrexate (MTX) is widely used in the treatment of ALL during the consolidation period. Assuming that toxicity induced by this chemotherapeutic regimen can be related to variants in genes involved in MTX metabolism, the identification of these genetic changes could contribute to the individualisation of chemotherapy to reduce the toxicity.[4]

The metabolic pathway of MTX is depicted in Figure 1. The cellular uptake of MTX occurs by passive diffusion, which is dependent on increasing extracellular

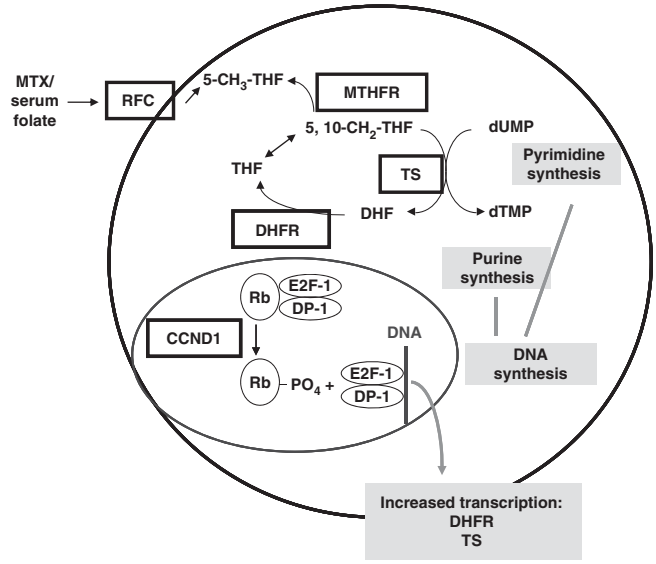

Figure 1 Scheme of the folate metabolism. DHF, dihydrofolate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; THF, tetrahydrofolate; 5-CH_3-THF, methyltetrahydrofolate; 5,10-CH_2-THF, methylenentetrahydrofolate. Genes analysed are shown in rectangles: transporter protein reduced folate carrier 1 (RFC1), regulatory enzyme 5,10-methylenentetrahydrofolate reductase (MTHFR), drug target thymidylate synthase (TS) and dihydrofolate reductase (DHFR). The role of the CCND1 in the transcription regulation is depicted in the inner diagram. DP-1, DP transcription factor 1; E2F-1, E2F transcription factor 1; MTX, methotrexate; Rb: retinoblastoma.

concentrations of MTX and also on a carrier transport mechanism mediated by reduced folate carrier 1 (RFC1). A common genetic polymorphism, consisting of a G>A transition in exon 1 of the *RFC1* gene, which replaces Arg by His, has been associated with higher serum MTX levels and worse prognosis in patients with *RFC1* 80AA genotype.[5]

Once inside the cell, MTX is polyglutamylated by the enzyme folylpolyglutamate synthetase. MTX and its polyglutamates are potent inhibitors of dihydrofolate reductase (DHFR). Moreover, MTX polyglutamates also target thymidylate synthase (TS). Inhibition of these enzymes affects DNA synthesis and cell replication, thereby blocking cell growth.

DHFR catalyses the conversion of dihydrofolate into tetrahydrofolate (THF). Its inhibition therefore depletes reduced forms of THF required in folate homeostasis. Recent studies have investigated the polymorphisms located in the promoter and gene coding regions in ALL paediatric patients. They found an association of the A allele of G308A variation and the *1b haplotype with a lower EFS. This association was more significant in high-risk patients.[6,7]

TS catalyses the nonreversible methylation of deoxyuridine-5-monophosphate to deoxythymidine-5-monophosphate, a precursor for DNA synthesis. Therefore, the inhibition of TS depletes thymidine and damages DNA. The *TS* gene is polymorphic, with either double (2R) or tri-tandem (3R) repeats of a 28-bp sequence in the promoter region. Homozygous 3R/3R cells over-express TS mRNA compared with homozygous 2R/2R cells.[8,9] A single-nucleotide polymorphism (SNP) G>C has been described at the 12th nucleotide of the second repeat of the 3R allele, leading to a tri-allelic locus (2R, 3RG and 3RC). This G>C substitution changes a critical residue in the upstream transcription factor E-box consensus element, abolishes upstream transcription factor-1 binding and alters transcriptional activity.[10] ALL children who are homozygous for the 3R allele have been found to have poorer EFS than those with other genotypes.[11,12]

Cyclin D1 (CCND1) can be associated with DHFR and TS regulation at the transcription levels. The transcription factors E2F-1 and DP-1 are released by the action of CCND1, a protein involved in the retinoblastoma protein phosphorylation, provoking an increase in the transcription levels of DHFR and TS.[13] The pharmacogenetic role of the polymorphism A870G in *CCND1* has been studied in childhood ALL patients and a lower EFS in homozygous cases for the A allele has been found.[14]

As shown in Figure 1, the 5,10-methylenetetrahydrofolate reductase (MTHFR) catalyses the reduction reaction of 5,10-methylene-THF to 5-methyl-THF. *MTHFR* C677T and A1298G polymorphisms have been associated with decreased activity of MTHFR and with increased levels of homocysteine.[15,16] Contradictory results linking these polymorphisms with relapse and toxicity risk in paediatric ALL patients have recently been reviewed.[17]

We investigated the pharmacogenetic role of several polymorphisms in genes involved in MTX metabolism. We also studied the usefulness of the *MTHFR* genotype to individualise high-dose MTX in the consolidation phase of childhood ALL therapy in an attempt to improve outcome without increasing toxicity.

Patients and methods

Patients

A total of 18 medical centres in Spain agreed to participate in the study. Of 499 Spanish children (aged 1–18 years) with newly diagnosed non-B ALL and treated with the ALL/SHOP-2005 protocol, 141 were eligible for pharmacogenetic studies. These patients were recruited from April 2005 to November 2009.

This protocol was approved by the Medical Ethics Committee at Hospital de la Santa Creu i Sant Pau. Informed consent was obtained before sample collection was performed.

Clinical data

ALL was diagnosed by morphological FAB criteria and immunophenotype. Central nervous system involvement was diagnosed if $\geqslant 5$ cells per μl were counted in the central nervous system in an atraumatic sample with identifiable lymphoblasts. Complete remission was considered when $<5\%$ lymphoblasts were present in the bone marrow without central nervous system involvement or leukaemic infiltration elsewhere.

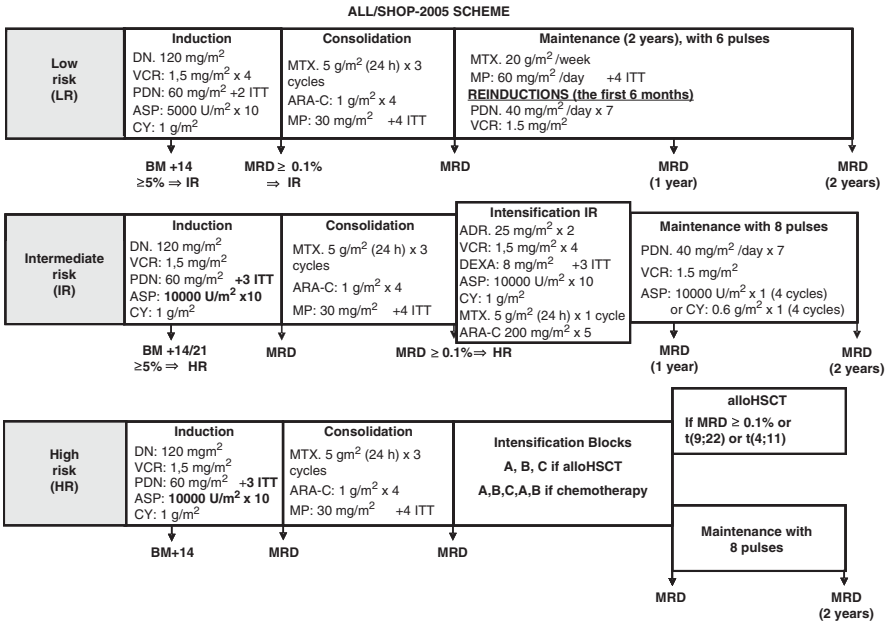

Figure 2 Protocol ALL/SHOP-2005 scheme. ALL, acute lymphoblastic leukaemia; alloHSCT, allogeneic haemopoietic stem cell transplantation; ARA-C, cytarabine; ASP, asparaginase; BM, bone marrow; CY, cyclophosphamide; DEXA, dexamethasone; DN, daunorubicin; HR, high-risk patient; IR, intermediate-risk patient; ITT, intrathecal therapy; LR, low-risk patient; MP, mercaptopurine; MRD, minimal residual disease; MTX, methotrexate; PDN, prednisone; VCR, vincristine.

Patients were stratified into three risk groups: low, intermediate and high risk according to a score based on age, immunophenotype, white blood cell count, extramedullary involvement, cytogenetic and molecular abnormalities, bone marrow blast cells at day 14 of treatment and presence of minimal residual disease at the end of induction.

The treatment protocol of ALL/SHOP-2005, schematised in Figure 2, consists of an induction and consolidation phase with prophylaxis of central nervous system disease. An early intensification phase is administered in intermediate-risk patients and five intensification blocks are administered in high-risk patients. A 2-year maintenance treatment is established for all patients except for those in the high-risk group who will receive allogeneic haemopoietic stem cell transplantation.

Haematological (leucopenia, anaemia, thrombocytopenia) and non-haematological (hepatic and gastrointestinal) toxicity was graded according to World Health Organization criteria (grades 0–4).

Genotype analyses

The genomic DNA was extracted from blood samples by the salting-out procedure.[18] The genetic markers analysed in the study are shown in Table 1.

We analysed the two SNPs (C677T and A1298T) in the *MTHFR* gene, the A80G polymorphism in the *RFC1* gene, the A870G polymorphism in the *CCND1* gene, as well as the A-317G and the C-680A promoter changes in the *DHFR* gene using the 48.48 dynamic array chips in the BioMark™ system (Fluidigm, South San Francisco, CA). This technology is designed for the allelic discrimination 5′ nuclease assay. The samples and the TaqMan SNP genotyping assay mixes were prepared following the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). The chips were primed in the NanoFlex IFC Controller (Fluidigm). Samples and genotyping assays mixes were then loaded into the inlets of the chips and the chips were returned to the IFC Controller for loading and mixing for 45 min. PCR was performed in the BioMark system (Fluidigm) and the thermal cycling conditions were: an initial step at 50 °C for 2 min and at 95 °C for 10 min, followed by a second step of 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Finally, the end point fluorescent image data were acquired on the BioMark Real-Time PCR System and analysed using the Fluidigm SNP Genotyping Analysis software (Fluidigm). Normal, heterozygous and homozygous sequenced samples of each polymorphism were included as internal controls.

Two additional polymorphisms in the *DHFR* gene were analysed: (i) C-1610G/T was determined by direct sequence using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), and (ii) a 19 bp insertion/deletion was determined by a conventional PCR method.[6]

In the *TS* gene, we analysed the variable number tandem repeat of 28 bp polymorphism and the G > C SNP in the first and second repeats. A DNA fragment was amplified using previously described PCR conditions and primers,[8] and directly sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). *TS* genotypes of the patients were classified into two groups according to Kawakami and Watanabe[19]: high-expression type (*2/*3G, *3C/*3G and *3G/*3G) and low-expression type (*2/*2, *2/*3C and *3C/*3C).

Table 1 Genetic markers and their frequencies in 141 ALL children

	(%)
MTHFR C677T, rs1801133	
C/C	41.8
C/T	49.6
T/T	8.5
MTHFR A1298C, rs1801131	
A/A	61.7
A/C	33.3
C/C	5.0
RFC1 A80G, rs1051266	
A/A	29.2
A/G	41.6
G/G	29.2
CCND1 A870G, rs603965/rs9344	
A/A	28.5
A/G	43.8
G/G	27.7
DHFR19 pb ins/del, rs70991108	
Ins/ins	30.9
Ins/del	52.9
Del/del	16.2
DHFR A-317G, rs408626	
A/A	32.1
A/G	51.1
G/G	16.8
DHFR C-680A, rs442767	
A/A	52.9
A/C	22.8
C/C	24.3
DHFR C-1610T/G, rs1650694	
C/C	38.3
C/G	30.1
C/T	9.0
G/G	9.0
G/T	12.8
T/T	8.0
TS VNTR 28pb (3′ region) and C>G	
High expression	45.6
Low expression	54.4

Abbreviations: ALL, acute lymphoblastic leukaemia; CCND1, cyclin D1; DHFR, dihydrofolate reductase; MTHFR, methylenetetrahydrofolate reductase; RFC1, reduced folate carrier 1; TS, thymidylate synthase; VNTR, variable number tandem repeat.

MTHFR *genotype/MTX dosage in the consolidation phase*

According to the treatment protocol, patients with an unfavourable *MTHFR* genotype (homozygous 677T, homozygous 1298C and compound heterozygous patients) associated with a decreased enzymatic activity were given $3\,g\,m^{-2}$ MTX in a 24-h infusion, whereas patients with a favourable *MTHFR* genotype (heterozygous and wild-type patients) associated with a normal enzymatic activity were given an increased dose of $5\,g\,m^{-2}$ MTX.

Statistical analysis

We performed the following statistical analyses to study whether MTX dose during consolidation therapy can be increased without increasing toxicity in patients with a *MTHFR* genotype associated with a normal enzymatic activity. The χ^2 test (univariate analysis) was used to estimate the risk of developing toxicity after therapy in patients with different genotypes. Multivariate logistic regression analysis was used to calculate the adjusted odds ratio and the dependent variable was the degree of toxicity. The multivariate model included the risk groups and the polymorphisms as covariates.

We used the following parameters to study the association between EFS and genotype. EFS was measured from the date of diagnosis to the date of event (recurrence, death or last follow-up). For censored cases, EFS corresponded to the time between diagnosis and the end of follow-up. Survival rates and median survival times were estimated using the Kaplan–Meier method with the log-rank test to determine statistical significance. Cox's regression analysis was used to estimate the hazard ratio (HR) for developing an event in the follow-up period (95% confidence interval). Clinical risk groups and other polymorphisms were included as covariates.

Bonferroni multiple comparison corrections considering four genetic variables has been applied and differences between means were considered statistically significant if the *P*-value was <0.013.

All analyses were performed by SPSS Statistical Package (SPSS, Chicago, IL, USA).

Results

A total of 141 patients with childhood ALL were treated with ALL/SHOP-2005 protocol according to their risk assessment (see Methods). Their demographic/clinical characteristics are summarised in Table 2.

The frequencies of the different alleles defined by the polymorphisms included in the study are shown in Table 1; all of them were in Hardy–Weinberg equilibrium.

Two variations in the *DHFR* gene, A-317G and 19 pb ins/del, were in linkage disequilibrium ($r^2=0.956$; minor allele frequency $=0.05$): alleles with an A residue in the A-317G SNP contained an insertion in the 19 pb ins/del polymorphism, and alleles with a G in the A-317G SNP contained a deletion in the 19 pb ins/del polymorphism. As both variations gave almost the same information for association analysis, from here on, we consider only the role of 19 pb ins/del polymorphism.

Analysis of toxicity in association with the polymorphisms affecting folate metabolism

Only the most severe adverse effects that appeared in any of the three high-dose MTX administrations in the consolidation phase were taken into account.

We have previously defined the combination of *MTHFR* polymorphisms that define the genotypes associated with a decreased enzymatic activity (unfavourable *MTHFR* genotype) and those associated with a normal activity (favourable *MTHFR* genotype).[20] The use of this *MTHFR* genetic classification in the analysis of the toxicities developed by our patients showed that there are two statistically significant associations: (i) between a significant decrease in the number of platelets ($<50\times10^9\,l^{-1}$) and the presence of a genotype associated with a decreased MTHFR activity (34.3% vs 14.3%; $P=0.014$) and (ii) between a high level of serum creatinine ($>1.26\times N^*\,\mu mol\,l^{-1}$) and the same *MTHFR* genotype (18.2 vs 3.9%; $P=0.013$).

Table 2 Patients' characteristics in ALL/SHOP-2005 pharmacogenetic study

	n	*(%)*
Gender		
Male	84	59.6
Female	57	40.4
Median age	5 years (range 1–18)	
WBC		
$<20\times10^9\,l^{-1}$	88	62.4
$20-200\times10^9\,l^{-1}$	44	31.2
$>200\times10^9\,l^{-1}$	9	6.4
Immunophenotype		
T	18	12.8
Non-T	123	87.2
Risk categories		
Low	37	26.2
Intermediate	85	60.3
High	19	13.5
CNS leukaemia		
No	139	98.6
Yes	2	1.4

Abbreviations: ALL, acute lymphoblastic leukaemia; CNS, central nervous system; WBC, white blood count.

Analysis of the remaining polymorphisms and their relationship with the development of toxicities has shown that: (i) thrombocytopenia is associated with the genotype ins/ins of the 19 pb ins/del polymorphism in the *DHFR* gene (36.4 vs 10.9%; $P=0.001$) and (ii) grade $\geqslant2$ mucositis was associated with the genotype G/G of the *RFC1* gene (25.6 vs 7.4%; $P=0.008$).

Multivariate analysis also considered the clinical risk categories as a covariate and showed that only the following associations maintained their statistical significance: thrombocytopenia $<50\times10^9\,l^{-1}$ with a genotype associated with a decreased MTHFR activity ($P=0.006$, HR: 4.06, confidence interval (CI): 95% 1.5–11.1); thrombocytopenia $<50\times10^9\,l^{-1}$ with the G/G genotype of the *RFC1* gene ($P=0.02$, HR: 3.2, CI: 95% 1.2–8.6) and thrombocytopenia $<50\times10^9\,l^{-1}$ with the genotype ins/ins of the 19 pb ins/del polymorphism in the *DHFR* gene ($P=0.002$, HR: 4.7, CI: 95% 1.8–12.6). Severe neutropenia was associated with the C/C genotype in the C-680A SNP of the *DHFR* gene ($P=0.01$, HR: 2.9, CI: 95% 1.3–7.1). The high level of serum creatinine ($>1.26\times N^*\,\mu mol\,l^{-1}$) was associated with the *MTHFR* genotype that determined a decreased enzymatic activity ($P=0.013$, HR: 5.5, CI: 95% 1.4–20.9). Finally, the relationship between grade $\geqslant2$ mucositis and the genotype G/G of the *RFC1* gene remained significant ($P=0.008$, HR: 4.4, GI: 95% 1.4–13.2).

Event-free survival and genotype

In relation to clinical and genetic determinants, the univariate analysis of EFS demonstrated significant differences between patients grouped in accordance with the clinical classification ($P=0.047$; Figure 3a). The *MTHFR* genotype classified the patients into two groups with significantly different EFS at 48 months ($P=0.012$; Figure 3b). The other genetic markers did not reveal differences. Using the Cox regression model, which includes both clinical and genetic determinants, the clinical classification and the *MTHFR* genotype retained their independent predictive value (Table 3). EFS was similar in low- and intermediate-risk cases, whereas it was significantly lower in high-risk patients. Patients with a *MTHFR* genotype associated with decreased enzyme activity were 4.3 times more at risk to suffer an event than those with a genotype

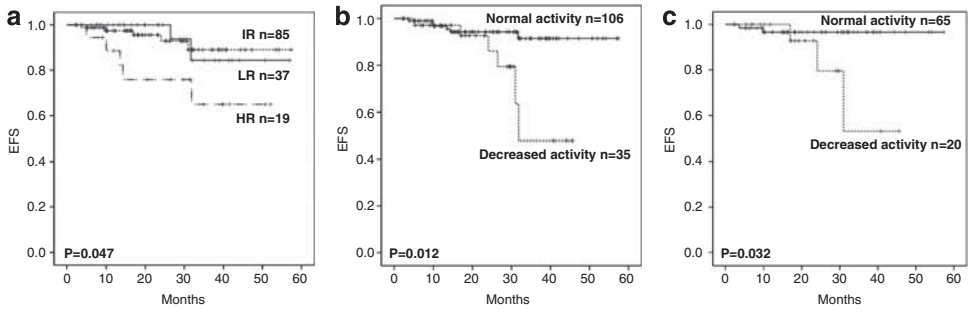

Figure 3 Kaplan–Meier estimates of event-free survival according to: (**a**) clinical classification (LR, low-risk patients; IR, intermediate-risk patients; HR, high-risk patients), (**b**) *MTHFR* genotype associated with a normal enzymatic activity and with a decreased activity and (**c**) *MTHFR* genotype in intermediate-risk patients.

Table 3 Cox regression analysis of EFS

Variable	P-*value*	*HR (CI 95%)*
Clinical classification	0.027	
Intermediate vs low-risk	0.9	—
High vs low-risk	0.04	6.9 (1.1–43)
MTHFR genotype associated with decreased vs normal activity	0.016	4.3 (1.3–14.0)

Abbreviations: CI, confidence interval; EFS, event-free survival; HR, hazard ratio; MTHFR, methylenetetrahydrofolate reductase.

associated with normal MTHFR activity. The genotype effect was especially relevant in the group of patients with a clinical intermediate-risk (Figure 3c).

Discussion

In this study, we found that children with a favourable *MTHFR* genotype treated with MTX doses of 5 g m^{-2} develop a similar or, in some cases, less toxicity than those children with an unfavourable genotype that were treated with the classical MTX dose of 3 g m^{-2}. An increase in the MTX dose from 3 to 5 g m^{-2} in the consolidation phase was the most relevant change introduced in the ALL/SHOP-2005 protocol used in the present study with respect to the previous ALL/SHOP protocols.[21] As there are data that emphasise the pharmacogenetic role of the *MTHFR* genotype in treatments including MTX, we decided to increase the MTX dose only in those patients with a *MTHFR* genotype associated with normal enzymatic activity. In the group of patients with a genotype associated with decreased MTHFR activity, we administered the same dose of MTX as that used in the previous ALL/SHOP protocols: 3 g m^{-2} in a 24-h infusion.

We also found that ALL children with a favourable *MTHFR* genotype treated with MTX doses of 5 g m^{-2} had significantly better EFS at 48 months. Other studies have also linked the *MTHFR* genotype with outcome in this setting. In a cohort of 201 children treated with different ALL protocols, Krajinovic *et al*.[22] showed that patients with the *MTHFR* T677A1298 haplotype had a poorer EFS. In a trial of 520 patients treated on CCG-1891 study, Aplenc *et al*.[23] found that the *MTHFR* C677T polymorphism was associated with relapse but the *MTHFR* A1298G polymorphism was not. More recently, Pietrzyk *et al*.[24] examined the relation between the *MTHFR* gene and mortality risk in children treated with the ALL IC BFM 2002 protocol, and found that the *MTHFR* 677TT genotype was fourfold more frequent in the children who died (31/389).

The main adverse effect of MTX is nephrotoxicity. MTX and its metabolite 7-hydroxy-MTX precipitate in the renal tubules causing renal dysfunction.[25] This, in turn, may delay MTX excretion and maintain high plasma MTX levels, increasing toxicity even further. Even though patients are monitored and precautions such as hydration, alkalinisation of urine and leucovorin rescue are taken to prevent renal toxicity, nephrotoxicity continues to occur in some patients. Turello *et al*.[26] recently reported the case of a child with lymphoblastic T-cell lymphoma and homozygous for *MTHFR* genotype who had high levels of serum creatinine after MTX treatment. Gammon *et al*.[27] reported the case of an adult patient with large B-cell lymphoma who was heterozygous for this genotype and also had high creatinine levels. The authors considered that toxicity appeared because of a marked folate depletion caused by the combined effect of MTX and the *MTHFR* genotype. Our results support these findings in that patients with the *MTHFR* genotype associated with a decreased enzymatic activity had statistically significant higher levels of creatinine in serum, despite of the reduced high-dose MTX (3 g m^{-2}).

Haematological toxicities, particularly thrombocytopenia, may also occur because of persistently high serum MTX concentrations. A recent study in patients treated for childhood ALL and non-Hodgkin lymphoma with high-dose MTX (5 g m^{-2}) reported that subjects with *MTHFR* polymorphism for A1298C had significantly higher MTX levels at 48 h and presented more haematological toxicities, including thrombocytopenia ($P = 0.0001$).[28] Our results also showed a significant decrease in the number of platelets in patients with a *MTHFR* genotype associated with a decreased enzymatic activity.

Data concerning the involvement of polymorphisms in other genes in the MTX pathway are scarce and in some cases, contradictory. We have found the following phenotype–genotype associations: (i) ins/ins of the 19 pb ins/del polymorphism in the *DHFR* gene with thrombocytopenia, (ii) the genotype G/G of the *RFC1* gene with mucositis grade $\geqslant 2$. Further prospective studies with larger number of cases are needed to establish the clinical usefulness of these findings.

In summary, our results show that the dose of MTX can be increased up to 5 g m^{-2} in the consolidation phase of ALL treatment without increasing toxicity in patients with a favourable *MTHFR* genotype. These findings therefore indicate that analysis of the *MTHFR* genotype is a useful tool to optimise MTX therapy in childhood patients with ALL. The application of 'risk-adapted' therapy represents an important advance in the treatment of childhood ALL. The identification of pharmacogenetic markers of interest in antileukemic treatments will provide refinement in risk classifications schemes allowing optimal treatment selection and dose individualisation in ALL children.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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