Folate cycle gene variants and chemotherapy toxicity in pediatric patients with acute lymphoblastic leukemia

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The gene polymorphisms of the methotrexate (MTX) action pathway influence event-free survival (EFS) in children with acute lymphoblastic leukemia (ALL). Here we assessed whether the gene variants associated with lower EFS also correlate with lower rates of episodes of toxicity. Homozygous individuals for cyclin D1 (*CCND1*) A870 allele and carriers of at least one methylenetetrahydrofolate reductase (*MTHFR*) T_{677} variant had a significantly lower frequency of weeks with high-grade hematologic and liver toxicity during consolidation and maintenance treatment, as based on the analysis of 186 pediatric ALL patients. This finding may have importance for MTX dose adjustment.

Key words: pharmacogenetics, methotrexate, polymorphisms, ALL, response to treatment, toxicity.

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Methotrexate (MTX) is an important component of consolidation and maintenance therapy of childhood acute lympoblastic leukemia (ALL). Nevertheless, a certain number of patients can develop resistance or adverse drug effects, which may hamper the efficacy of treatment or require drug dose reduction and drug withdrawal. Following the hypothesis that gene variants of the MTX action pathway can affect outcome of ALL, we analyzed a number of polymorphisms in the genes of the folate cycle for their impact on reduced event-free survival (EFS). Thymidylate synthase (TS) is inhibited by glutamylated forms of MTX.[1] A repeat polymorphism is described in the enhancer region of this gene with a triple repeat (3R) increasing gene transcription and TS levels.[2] A G80A polymorphism of the reduced folate carrier (*RFC1*) gene, encoding a major MTX transporter, results in an amino-acid replacement in the RFC1 domain considered important in folate/antifolate binding.[3] The A870G polymorphism in the cyclin D1 (*CCND1*) modulates mRNA splicing[4] and altered CCND1 expression was previously shown to play a role in the development of MTX resistance.[5] The substrate for methylene tetrahydrofolate reductase (MTHFR), 5,10-methylene-tetrahydrofolate, and its derivate, 10-formyl tetrahydrofolate, whose formation is dependent on the methylene tetrahydrofolate dehydrogenase (MTHFD1), are essential cofactors for *de novo* thymidylate and purine synthesis.[1] Two polymorphisms in the *MTHFR* gene, a $C_{677}T$ and an $A_{1298}C$ substitution, result in amino-acid replacements causing reduced enzymatic activity, whereas a G1958A substitution in the *MTHFD1* gene leads to an amino-acid change within the 10-formyl-tetrahydrofolate synthetase domain.[6-8] Recently we described reduced EFS for childhood ALL patients who are homozygous for the *TS* $3R^9$ or *CCND1* A870 variant[10] as well as for carriers of at least one *MTHFR* T677[11] or *RFC1* A_{80} allele.[12] The role of *TS* 3R and *CCND1* A_{80} homozygosity, as well as that of *MTHFR* T677 allele in poorer childhood ALL outcome was recently confirmed by others.[13-15] Here we hypothesized that variants associated with reduced EFS can also correlate with a lower incidence of toxicity in patients with childhood ALL.

Design and Methods

The ALL patients were treated at Sainte-Justine Hospital with one of three treatment protocols of the Dana-Farber Cancer Institute (87-01, 91-01 and 95-01). Data were available for 186 patients out of 200 consecutive patients, all of European descent (age range 1-18 years), who were previously assessed for the relationship between genotypes and EFS. These patients and their disease characteristics, as well as details of treatment have been previously described.[12] In brief, MTX was given once a week at a

dose of 30 mg/m^2 during consolidation and maintenance in all three protocols. Dose modification guidelines were also the same across the three protocols.

Estimates of toxicity to bone marrow and liver function were based on the reduction of white blood cells (leukopenia), platelets (thrombocytopenia) and absolute neutrophil count, and elevation of liver enzymes (alanine aminotrasferase and aspartate aminotrasferase). Laboratory tests were performed weekly, 7 days after MTX administration. The mean number of weeks assessed per patient was 80. Toxicity was graded using the common criteria for adverse events of the National Cancer Institute. Grades 3 and 4 were considered for all parameters except for thrombocytopenia for which grade 2 was also included. Thrombocytopenia grade 3 and 4 or liver toxicity grade 4 occurred rarely, and in such cases grades 3 and 4 were combined whereas the remaining parameters were considered separately for the analysis (Table 1). Previously obtained genotypes were used for the analysis; the details of the genotyping have been described elsewhere.[9-12] Information on MTX dose during maintenance treatment was available in the same patients and the correlation between drug doses and genotypes was reported previously.[10,11]

This study was approved by the Ethics Committee of Sainte-Justine Hospital and the research was conducted in accordance with the Declaration of Helsinki.

Statistics

Genotypes are analyzed as dichotomous variables in accordance with their influence on EFS. The genotype grouping includes either carriers of minor alleles (*MTHFR* T_{677}, *MTHFR* C_{1298}, *MTHFD1* A_{1958} and *RFC1* A_{80} variants) or homozygous individuals for *TS* 3R and *CCND1* A_{870} allele who were compared to patients without these genotypes. The frequencies of weeks with high-grade hematologic and liver toxicity was compared using the Mann-Whitney test. The proportion of weeks with a particular toxicity between all patients with and without given genotypes was compared using the χ^2 test, and accompanied by the genotype-associated rate ratio.

Results and Discussion

The frequencies of weeks with high-grade hematologic and liver toxicity that developed following MTX administration during consolidation and maintenance treatment are outlined in Table 1. The inter-patient variability is shown by the large variance. The comparison of the frequencies of these toxicities between individuals with and without indicated genotypes showed that individuals with *MTHFR* T_{677} allele had significantly lower rates of grade 3 leukopenia and individuals with *CCND1* AA_{870} genotype had lower rates of grade 3 leukopenia, grade 2 thrombocytopenia and grade 3/4 liver toxicity, as estimated by increased alanine aminotrasferase levels (Table 2). Likewise, when the proportion of weeks with these toxicities was compared between groups with and without the *MTHFR* TT/CT_{677} or *CCND1* AA_{870} genotype, highly significant results were obtained showing a lower proportion of weeks with the toxicity among individuals with these genotypes. Accordingly, the toxicity rate-ratio associated with the genotype was lower, in most cases showing 2-fold reductions (Table 2). When the combined effect of *MTHFR* and *CCND1* genotypes was assessed, the frequency of weeks with grade 3 leukopenia was further decreased in individuals with both *CCND1* AA_{870} genotype and *MTHFR* T_{677} allele, and increased in individuals without these genotypes (Table 3). There were not significant correlations with the other polymorphisms analyzed.

We previously observed that ALL patients with *MTHFR* TT/CT_{677} or *CCND1* AA_{870} genotypes tended to have, or, as in case of *CCND1*, had a significantly lower frequency of weeks with MTX dose reduction or withdrawal.[10,11] However, the average dose of MTX received weekly did not differ across different genotypes of the same polymorphism and was lower than the maximal dose predicted by the protocol leaving the possibility for drug dose adjustment. Here we extended the analysis beyond drug dose by analyzing the relationship between genotypes and the frequency of hematologic and liver toxicity during consolidation and maintenance treatment. Individuals with *CCND1* AA_{870} or MTHFR

Table 1. The frequency of high-grade hematologic and liver toxicity during consolidation and maintenance treatment of 186 ALL patients.

Toxicity‡	*N (%)*	*Frequency (%)*			
		mean	*median*	*SD*	*maximum*
WBC 3 (1-2×10^9/L)	129 (69.4)	4.4	2.5	5.8	36.1
WBC 4 (<1×10^9/L)	16 (8.6)	0.2	*	0.7	6.7
ANC 3 (0.5-1×10^9/L)	173 (94)	9.1	8.0	7.0	35
ANC 4 (<0.5×10^9/L)	115 (61.8)	2.4	1.3	3.0	15.5
PLT 2 (50-75×10^9/L)	20 (10.8)	0.2	*	0.6	4.8
PLT 3/4 (<50×10^9/L)	13 (7.0)	0.1	*	0.5	3.3
ALT 3/4 (>5×ULN)	116 (62.4)	3.0	1.6	3.8	17.5
AST 3/4 (>5×ULN)	59 (31.7)	0.7	*	1.5	8.2

*N (%), number and frequency of patients with at least one episode of the indicated toxicity. Frequency (%), overall frequency of weeks with indicated toxicities; average values per patient are given. ‡Toxicity was graded using the common criteria for adverse events (in parenthesis) of the National Cancer Institute, version 3.0 (http://ctep.cancer.gov/reporting/ctcnew.html). SD, standard deviation; * not calculated due to rare occurrence; WBC, white blood cell count; WBC3 and WBC 4, leukopenia grade 3 and grade 4; ANC, absolute neutrophil count; ANC3 and ANC4, neutropenia grade 3 and grade 4; PLT, platelets; PLT 2, thrombocytopenia grade 2; PLT 3/4, thrombocytopenia grade 3 and 4 combined; ALT alanine aminotrasferase; AST, aspartate aminotransferase; ALT 3/4 and AST 3/4, elevation of ALT and AST levels to values more than five times higher than upper limit of normal (ULN), corresponding to grade 3 and 4 toxicity combined.*

Table 2. The relationship between genotypes and the parameters of high-grade toxicity.

Toxicity	Descriptive statistics*	Genotype		p and RR‡
		MTHFR CT/TT	*MTHFR CC*	
	individuals	108	78	
	Total n. of weeks	8635	6326	
WBC 3	Total n. of weeks with toxicity	300	349	*p*1<0.0001 RR=0.6, 95 % CI=0.5-0.7
	median %	1.8	3.5	*p*2=0.03
		CCND1 AA	*CCND1 GG/GA*	
	N. of individuals	44	142	
	Total n. of weeks	3519	11441	
WBC 3	Total n. of weeks with toxicity	101	548	*p*1<0.0001 RR=0.6, 95 % CI=0.5-0.8
	median %	1.3	3.1	p2=0.05
PLT 2	n. of weeks with toxicity	1	23	p1=0.05 R=0.1, 95 % R CI=0.02-1.0
	mean %†	0.03	0.2	*p*2=0.04
ALT3/4	Total n. of weeks with toxicity	74	355	*p*1=0.002 RR 0.7, 95 % CI=0.5-0.9
	Median %	1.0	2.0	*p*2=0.03

*MTHFR: methylene tetrahydrofolate reductase; CCND1: cyclin D1; WBC3: grade 3 leukopenia; PLT2: grade 2 thrombocytopenia; ALT 3/4: elevation of ALT levels to toxicity grade 3 and 4 combined. *Total weeks with toxicity reflects the sum of weeks with the given toxicity for all patients with indicated genotype. Median % reflects the median frequency of weeks with the given toxicity per patient/per genotype; †median could not be calculated due to the limited number of patients; the mean value is given instead. ‡p1 is obtained by the χ^2 test and p2 by Mann-Whitney; RR, rate ratio.*

Table 3. The relationship between combined MTHFR and CCND1 genotypes and the reduction in white blood cell count.

Toxicity	Descriptive statistics *	Genotype		p and RR‡
		MTHFR CT/TT CCND1 AA	*MTHFR CC CCND1 GG/GA*	
	N. of individuals	27	61	
	Total n. of weeks	2125	4932	
	Total n. of weeks with toxicity	56	304	*p*1<0.0001 RR= 0.4, 95 % CI =0.3-0.6
WBC 3	median %	1.0	4.0	*p*2=0.01

*MTHFR: methylene tetrahydrofolate reductase; CCND1: cyclin D1; WBC 3: grade 3 leukopenia. * total N weeks with toxicity reflects the sum of weeks with grade 3 leukopenia for all patients with indicated genotype; Median % reflects the median frequency of weeks with leucopenia grade 3 per patient/per genotype. ‡p1 is obtained by the χ^2 test and p2 by the Mann-Whitney; RR, rate ratio.*

TT/CT$_{677}$ genotype had lower rates of these toxicities. As for ALL outcome, which was affected more substantially by combined at-risk genotypes than by either genotype alone,[10] grade 3 leukopenia was further decreased in individuals with a combination of the *CCND1* AA$_{870}$ and *MTHFR* TT/CT$_{677}$ genotypes. Both *CCND1* A$_{870}$ and *MTHFR* T$_{677}$ are common among Caucasians, with a frequency of ~40% and ~35 %, respectively.[4,6] The observed correlation for *CCND1* follows the pattern predicted from the results of studies associating *CCND1* AA$_{870}$ genotype with reduced EFS,[10,15] and is in agreement with the functional impact of this polymorphism.[4] The *CCND1* A$_{870}$G substitution modulates the ratio of *CCND1* mRNA isoforms. The transcript associated with the *CCND1* A allele results in a protein with a longer half-life[4] resembling CCND1 over-expression, which has previously been shown to lead to the increased expression of MTX targets and reduction of sensitivity to MTX.[5]

Reduced MTHFR activity caused by the *MTHFR* T$_{677}$ allele leads to higher 5,10-methylene-tetrahydrofolate levels which, could facilitate uridylate-thymidylate conversion by *TS*, reducing the rate of uracil misincorporation into DNA and resulting chromosome damage. This might decrease MTX efficacy explaining both the reduced rates of leukopenia found in this study and the reduced EFS in ALL patients found previously.[11,16] In agreement with this is the finding showing that cells transfected with T677 cDNA have decreased MTHFR activity resulting in accelerated cellular growth rate accompanied by decreased chemosensitivity to MTX.[17] Three other studies have addressed the impact of MTHFR on the toxicity in ALL patients. Among adults who underwent treatment with different protocols, doses and schedules of MTX administration, TT$_{677}$ homozygotes more frequently experienced MTX intolerance although it was not clear how the frequency of the episodes of toxicity were accounted for in the analysis.[18] Kishi *et al.*,[19] on the other hand found no association between the T677 allele and either seizures or thrombosis in childhood ALL patients. Likewise, Aplenc *et al.*[16] did not observe a correlation between *MTHFR* genotypes and various types of higher-grade toxicity (central nervous system toxicity, diarrhea, hyperbilirubinemia, neuropathy, stomatitis, raised transaminase levels or infection). As in this latter study, we did not observe an influence of *MTHFR* genotypes on the increase of liver enzyme levels, whereas the other toxicity end-points were not comparable. Chemotherapy toxicity did not differ between carriers of the various genotypes of the other genes analyzed here. The reason for this could be a lower relative importance of some of the genes analyzed. For example, the *MTHFD1* variant allele did not play an important role in ALL outcome when analyzed with other prognostic factors.[11] The *RFC1* G$_{80}$A polymorphism played a minor role when analyzed simultaneously with other polymorphisms relevant to ALL outcome.[10] On the other hand *TS* 3R homozygosity was clearly associated with poorer ALL outcome after adjustment for other prognostic factors and polymorphisms studied.[10] In this case it is possible that other types of toxicities than those analyzed here

are different between patients with different *TS* genotypes. For example, childhood ALL patients with the *TS* 3R allele were shown to be less prone to osteonecrosis.[20]

In conclusion, we found that the particular polymorphisms of the folate cycle that correlated with reduced EFS, possibly due to lower sensitivity to MTX, also correlated with lower rates of episodes of toxicity. Although these results should be further verified and the analyses extended to a larger group of patients, this finding opens the possibility of drug dose adjustment: patients who have lower EFS and a lower frequency of chemotherapy toxicity might benefit from an increase in drug dose.

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