Brief report

Effect of polymorphisms in folate-related genes on in vitro methotrexate sensitivity in pediatric acute lymphoblastic leukemia

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We studied whether common polymorphisms in genes involved in folate metabolism affect methotrexate (MTX) sensitivity. Ex vivo MTX sensitivity of lymphoblasts obtained from pediatric patients with acute lymphoblastic leukemia (ALL; n = 157) was determined by the in situ thymidylate synthase inhibition assay after either continuous (21 hours; $TSI_{50,\ cont}$) or short-term (3 hours; $TSI_{50,\ short}$) MTX exposure. DNA was isolated from lymphoblasts obtained from cytospin slides. Polymorphisms in methylenetetrahydrofolate reductase (*MTHFR* 677C>T, *MTHFR* 1298A>C), methionine synthase (*MTR* 2756A>G), methionine synthase reductase (*MTRR* 66A>G), methylenetetrahydrofolate dehydrogenase (*MTHFD*1 1958G>A), serine hydroxymethyl transferase (*SHMT*1 1420C>T), thymidylate synthase (*TS* 2R3R), and the reduced folate carrier (*RFC* 80G>A) were detected by polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) or real-time PCR. Patients with the *MTHFR* 1298AC variant or the *MTRR* 66 G-allele showed decreased in vitro MTX sensitivity measured under both test conditions. *SHMT*1 1420TT homozygotes only showed decreased MTX sensitivity in the $TSI_{50,\ cont}$. In conclusion, polymorphisms in the folate-related genes *MTHFR*, *MTRR*, and *SHMT*1 are related to MTX resistance in pediatric patients with ALL. (Blood. 2005;106:717-720)

Introduction

Reduced tetrahydrofolates (THFs) serve as cofactors that carry one-carbon moieties for (1) the synthesis of de novo purines and thymidylate (dTMP) and (2) remethylation of homocysteine to methionine (Figure 1). The antifolate methotrexate (MTX) and its active polyglutamate forms inhibit RNA and DNA synthesis in cells by inhibiting dihydrofolate reductase (DHFR) and thymidylate synthase (TS), respectively, 2 crucial enzymes in pyrimidine synthesis and folate recirculation (Figure 1). MTX is an important chemotherapeutic drug in the treatment of acute lymphoblastic leukemia (ALL) and other malignancies. Recent clinical and in vitro studies indicate that common polymorphisms in genes coding for enzymes involved in folate metabolism affect the sensitivity of patients to folate-based chemotherapeutic drugs such as MTX.[1-10] This may be a direct effect or indirect due to shifts in intracellular folate distribution.[9,11] We investigated whether common polymorphisms in folate-related genes influence (ex vivo) MTX sensitivity.

Study design

From newly diagnosed Western European pediatric patients with ALL, peripheral blood or bone marrow mononuclear cells were isolated with Ficoll Isopaque (> 80% leukemic cell enrichment) and MTX sensitivity was previously measured with the in situ thymidylate synthase inhibition assay (TSI).[12,13] In short, leukemia cells were incubated with different concentrations of MTX for 21 hours (TSI_{cont}) or for 3 hours followed by an 18-hour drug-free period (TSI_{short}), after which the degree of inhibition of TS was measured using [5-^{3}H]-2′-deoxycytidine as substrate.[12] TSI data were expressed as the concentration of MTX needed to inhibit 50% of the control TS activity (TSI_{50}). Due to limited cell numbers, of all 157 patients included in this study, the $TSI_{50,\ cont}$ was measured in 86 patients and the $TSI_{50,\ short}$ in 120 patients. DNA was obtained from unstained cytospin slides of 157 patients. Informed consent from each patient was obtained according to local procedures in accordance with the Declaration of Helsinki. Genomic DNA was isolated using the QIAmp DNA Mini Blood Kit (Qiagen, Vealo, Netherlands). PCR-RFLP was used to detect polymorphisms in methylenetetrahydrofolate reductase (*MTHFR* 677C>T, *MTHFR* 1298A>C),[14,15] methionine synthase (*MTR* 2756A>G),[16] serine hydroxymethyl transferase (*SHMT*1 1420C>T),[17] methylenetetrahydrofolate dehydrogenase (*MTHFD*1 1958G>A),[18] and the reduced folate carrier (*RFC* 80G>A).[19] Double (2R2R) or triple (3R3R) 28-bp tandem repeats in the promoter region of the thymidylate synthase gene (*TS* 2R3R) were visualized on agarose gel directly after the PCR reaction.[20] The polymorphism in the methionine synthase reductase gene (*MTRR* 66A>G) was determined with real-time PCR using hybridization probes and Lightcycler Technology (Roche, Mannheim, Germany). Because TSI data were not normally distributed, ln (natural logarithm)–transformed TSI values were used as outcome measure in univariate and multivariate regression analysis.

Results and discussion

For each genetic variant, the median (50th percentile) and interquartile range (25th-75th percentile) for the $TSI_{50,\ cont}$ and the $TSI_{50,\ short}$ is presented in Tables 1 and 2. Univariate regression analysis revealed *MTHFR* 1298AC ($P = .01$), *MTRR* 66AG ($P = .08$), and

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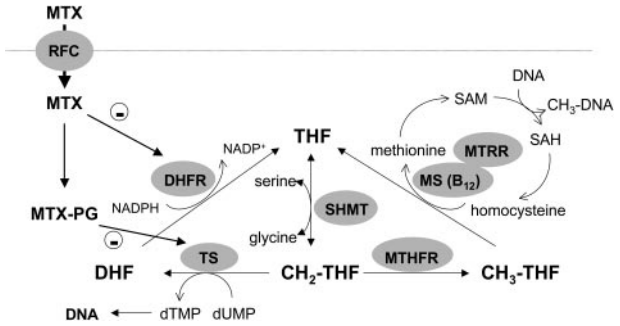

Figure 1. Simplified scheme depicting intracellular metabolism of folates. For clarity reasons, the reaction catalysed by methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase (MTHFD1) is not shown; MTHFD1 converts CH_2-THF in 3 steps to THF and provides formyl-THF (CHO-THF) for purine synthesis. THF indicates tetrahydrofolate; DHF, dihydrofolate; CH_2-THF, methylene-THF; CH_3-THF, methyl-THF; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; dTMP, deoxy thymidine monophosphate; dUMP, deoxy uridine monophosphate; TS, thymidylate synthase; MTHFR, methylenetetrahydrofolate reductase; MS, methionine synthase; MTRR, methionine synthase reductase; SHMT, serine-hydroxymethyltransferase; DHFR, dihydrofolate reductase; RFC, reduced folate carrier; MTX-PG, methotrexate-polyglutamate; and NADP, nicotinamide adenine dinucleotide.

MTRR 66GG ($P = .09$) as potential determinants of $TSI_{50,\ cont}$; *MTHFR* 1298AC ($P = .03$), *MTRR* 66AG ($P < .001$), and *MTRR* 66GG ($P = .02$) were determinants of $TSI_{50,\ short}$. Multiple regression analysis identified *MTHFR* 1298AC ($P = .06$), *MTRR* 66AG ($P = .08$), and *SHMT*1 1420TT ($P = .04$) as determinants of $TSI_{50,\ cont}$; determinants of $TSI_{50,\ short}$ were *MTHFR* 1298AC ($P = .01$), *MTRR* 66AG ($P = .001$), and *MTRR* 66GG ($P = .03$).

This is the first report describing the influence on a large population of several genetic polymorphisms in folate-related genes on ex vivo MTX sensitivity. Our results show that patients with the *MTRR* 66 G-allele and the *MTHFR* 1298AC variant are less sensitive to MTX in both the $TSI_{50,\ cont}$ and the $TSI_{50,\ short}$. Interestingly, both polymorphisms were also related to hematologic toxicity (manuscript in preparation). The *MTHFR* 677C>T polymorphism was not related to sensitivity in this study. In fact, published data on the role of *MTHFR* 677C>T are quite contradictory. Patients carrying the *MTHFR* 677C>T polymorphism are at a higher risk of developing MTX-related toxicity,[2,4,8] whereas in an earlier small pilot study (n = 6), greater MTX sensitivity was observed in the 2 *MTHFR* 677TT patients.[6] The *MTHFR* 677T1298A haplotype or the *MTHFD*1 1958A variant, in interaction with the TS 3R repeat, resulted in a decreased event- and

Table 1. Univariate analysis of the effect of polymorphisms in folate-related genes on in vitro MTX sensitivity (TSI_{50}) of childhood ALL

	Univariate analysis			
	$TSI_{50,cont}$, μM		$TSI_{50,short}$, μM	
Polymorphism	*n*	Median [IQR]	*n*	Median [IQR]
***MTHFR* 677C > T**				
CC	47	0.071 [0.041-0.195]	68	0.562 [0.156-4.978]
CT	31	0.063 [0.038-0.155]	39	0.540 [0.156-3.500]
TT	8	0.090 [0.037-0.168]	13	1.481 [1.037-3.250]
***MTHFR* 1298A > C**				
AA	36	0.052 [0.030-0.124]	51	0.600 [0.156-1.488]
AC	36	0.107 [0.055-0.225]†	49	0.904 [0.156-8.656]‡
CC	14	0.082 [0.034-0.194]	20	0.479 [0.156-3.449]
***MTR* 2756 A > G**				
AA	54	0.103 [0.035-0.180]	77	0.600 [0.156-6.059]
AG	26	0.052 [0.038-0.117]	36	0.661 [0.156-1.795]
GG	6	0.098 [0.056-0.204]	7	0.906 [0.156-1.460]
***MTRR* 66A > G**				
AA	23	0.054 [0.038-0.088]	29	0.156 [0.156-0.560]
AG	37	0.104 [0.038-0.191]§	54	1.260 [0.169-7.658]‖
GG	26	0.112 [0.038-0.190]¶	36	0.984 [0.156-4.802]‡
***MTHFD*1 1958G > A**				
GG	26	0.120 [0.033-0.196]	35	0.830 [0.156-2.840]
GA	48	0.065 [0.041-0.164]	67	0.762 [0.156-5.310]
AA	12	0.053 [0.036-0.174]	18	0.560 [0.156-2.653]
***SHMT*1 1420 C > T**				
CC	49	0.063 [0.037-0.161]	74	0.661 [0.156-4.096]
CT	30	0.067 [0.037-0.182]	35	0.600 [0.156-3.500]
TT	7	0.133 [0.088-0.235]	11	1.131 [0.410-5.614]
***TS* 2R3R**				
3R3R	25	0.059 [0.039-0.192]	42	0.951 [0.156-5.255]
3R2R	42	0.069 [0.035-0.132]	52	0.398 [0.156-2.963]
2R2R	19	0.151 [0.050-0.181]	25	1.042 [0.178-5.384]
***RFC* 80G > A**				
GG	30	0.058 [0.034-0.173]	43	0.580 [0.156-1.858]
GA	43	0.071 [0.036-0.188]	54	0.508 [0.156-3.620]
AA	12	0.080 [0.044-0.163]	20	1.110 [0.190-9.315]

IQR indicates interquartile range.
†$P < .01$.
‡$P < .05$.
§$P = .08$.
‖$P < .001$.
¶$P = .09$.

Table 2. Multivariate analysis of the effect of polymorphisms in folate-related genes on in vitro MTX sensitivity (TSI_{50}) of childhood ALL

	Multivariate analysis	
Polymorphism	$TSI_{50,cont}$, Stand β	$TSI_{50,short}$, Stand β
***MTHFR* 677C > T**		
CT vs CC	−0.033	−0.043
TT vs CC	0.044	0.067
***MTHFR* 1298A > C**		
AC vs AA	0.264*	0.281†
CC vs AA	0.064	0.074
***MTR* 2756 A > G**		
AG vs AA	−0.100	−0.047
GG vs AA	−0.005	−0.019
***MTRR* 66A > G**		
AG vs AA	0.272§	0.399‖
GG vs AA	0.204	0.272‡
***MTHFD*1 1958G > A**		
GA vs GG	0.012	0.137
AA vs GG	−0.060	−0.003
***SHMT*1 1420 C > T**		
CT vs CC	−0.002	0.026
TT vs CC	0.259‡	0.096
***TS* 2R3R**		
3R2R vs 3R3R	−0.088	−0.058
2R2R vs 3R3R	−0.034	0.012
***RFC* 80G > A**		
GA vs GG	0.043	−0.003
AA vs GG	0.009	0.057

The standardized regression coefficient of each independent variable is given as standardized β (Stand β) in multivariate analysis. Because independent variables may have different units, the strength of the regression coefficients (β) may not be comparable and therefore the coefficients are standardized to their means (Stand β) for comparison reasons. In univariate regression, the strength of the association between each independent variable and the dependent variable (outcome measure) is looked at without taking into account the other independent variables; in contrast, in multiple linear regression, the strength of each independent variable is adjusted by the other independent variables in the model. No significant interactions were observed in multiple regression analysis for MTHFR 677C>T*MTHFR 1298A>C, MS2756 A>G*MTRR 66A>G, MTHFR 677C>T*MTHFD1 1958G>A, and MTHFR 677C>T*MTHFD1 1958G>A.

*$P = 006$.
†$P < .01$.
‡$P < .05$.
§$P = .08$.
‖$P < .001$.

disease-free survival in pediatric patients with ALL treated with MTX.[10] Sohn et al[9] transfected human colon and breast cancer cells with either wild-type or mutant 677T human MTHFR cDNA. Only mutant cells had decreased MTHFR activity, changed intracellular folate distribution, increased TS activity, and decreased chemosensitivity toward MTX. Our study with clinical samples of childhood ALL could not confirm the influence of the *MTHFR* 677C>T polymorphism on ex vivo MTX sensitivity, although a trend toward decreased sensitivity was observed. Some of the discrepancies might be explained by the different conditions used for the in vitro assays. In our assay, high folate is present in the culture medium, possibly counteracting changed folate redistribution due to polymorphisms in folate genes. The clinical results are possibly explained by changed folate distribution. We have no explanation why only the heterozygous *MTHFR* 1298AC variant showed decreased MTX sensitivity whereas the homozygous (CC) variant did not.

Also, in other diseases in which MTX is being used, genetic polymorphisms play a role. *MTHFR* 1298A>C and not *MTHFR* 677C>T modulated the efficacy of MTX treatment in rheumatoid arthritis patients, whereas *MTHFR* 677C>T was related to toxicity.[3,5] Both *TS* 2R/3R[7,21] and *RFC* 80G>A[21] polymorphisms influenced efficacy and not toxicity of low-dose MTX therapy in patients with rheumatoid arthritis, whereas both *MTHFR* polymorphisms showed no effect.[7]

Our study is the first to report on the *MTRR* 66A>G and *SHMT*1 1420C>T polymorphisms as pharmacogenetic determinants of MTX treatment efficacy. Further studies are warranted to evaluate the effect of these polymorphisms on enzyme activity and on intracellular folate redistribution. However, increased risk of neural tube defect pregnancy outcome and cardiovascular disease have been associated with the *MTRR* 66A>G polymorphism.[22] *SHMT*1 1420CC individuals show increased homocysteine concentrations and lower folate status[22] and exhibit an increased ALL risk.[17] TS competes with SHMT and MTHFR for methylenetetrahydrofolate.[23] *MTHFR* 677C>T and *MTHFR* 1298A>C reduce in vitro enzyme activity.[14,15] *MTHFR* 677C>T directs methylenetetrahydrofolate toward purine and pyrimidine synthesis at the expense of methyltetrahydrofolate, and increases catalytic activity of TS.[9,11] SHMT mediates the competition between TS and MTHFR for one-carbon units: increased SHMT expression favors DNA synthesis and inhibits homocysteine remethylation and S-adenosylmethionine (SAM) synthesis.[24] MTRR maintains adequate levels of methyl-cobalamin, the activated cofactor of MS, and thus maintains MS in its active state. Low MTRR activity prevents MTR-mediated remethylation of homocysteine and, because the MTHFR reaction is irreversible, results in trapping of methyl-tetrahydrofolate with concomitant inhibition of purine and thymidylate biosynthesis.[23] We have no explanation why *SHMT*1 TT patients only showed increased MTX resistance in the $TSI_{50,\ cont}$.

In contrast to the $TSI_{50,\ short}$, borderline significances were observed for the $TSI_{50,\ cont}$ in our study. This may be due to the lower number of patients in which the $TSI_{50,\ cont}$ was measured ($n = 86$) compared with the $TSI_{50,\ short}$ ($n = 120$) with concomitant lower statistical power. More likely, however, accumulation of long-chain MTX polyglutamates is higher after continuous incubations ($TSI_{50,\ cont}$), resulting in an increased inhibition capacity of TS. As a consequence, $TSI_{50,\ cont}$ is less sensitive to changes in folate redistribution due to polymorphisms in other folate-related enzymes. The TSI correlates with conventional cytotoxic assays in leukemic cell lines and the $TSI_{50,\ short}$ gives a better reflection of clinical MTX sensitivity than the $TSI_{50,\ cont}$.[12]

In conclusion, we show that the common polymorphisms in the folate-related genes *MTHFR*, *MTRR*, and *SHMT*1 are related to in vitro MTX sensitivity in pediatric patients with ALL. To date, the MTX dose applied is based mostly on the body surface area, while the patient's cellular folate status or pharmacogenetic background[25] is not yet routinely taken into account. Identifying predictors of MTX sensitivity may lead to the development of individualized treatment strategies with improved efficacy and reduced toxicity. In some countries, but not The Netherlands, the TPMT polymorphism is used to dose adjust the *initial* dose of 6-mercaptopurine. We feel that in a similar way polymorphisms in folate-related genes such as MTRR could be used to adjust the initial (not maintenance) MTX dose once it is firmly established that these polymorphisms influence efficacy and/or toxicity. In 500 healthy white subjects, we established high allele frequencies of *MTRR* 66A>G (55%), *MTHFR* 1298A>C (31%), and *SHMT*1 1420C>T (32%). Athough ethnic and geographic variations exist in these polymorphisms, high allele frequencies have also been reported in other ethnic groups and thus, they may significantly contribute to sensitivity and toxicity associated with MTX treatment.

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