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ORIGINAL ARTICLE

The role of MTHFR and RFC1 polymorphisms on toxicity and outcome of adult patients with hematological malignancies treated with high-dose methotrexate followed by leucovorin rescue

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Abstract
Purpose In the last years, the influence of different genes involved in metabolism of chemotherapeutic agents has been studied. Methotrexate (MTX) is a key compound of chemotherapeutic regimens used in the treatment of acute lymphoblastic leukemia (ALL), primary central nervous system lymphoma (PCNSL) and Burkitt's lymphomas (BL). This study aims to evaluate the role of MTHFR C677T and A1298C polymorphisms and G80A reduced folate carrier gene (RFC1) in a cohort of adult patients with lymphoproliferative malignancies submitted to high-dose MTX followed by leucovorin rescue.
Methods We performed the analysis of these polymorphisms on genomic DNA with RFLP–PCR.
Results Patients carrying MTHFR A1298C variant showed decreased hepatic and hematological toxicity ($P = 0.03$). Overall survival (OS) and progression-free survival (PFS) between homozygous wild-type and variant patients for the RFC1 $G_{80}A$ were significantly different ($P = 0.035$ and $P = 0.02$, respectively). A significant correlation between hematological toxicity and age ($P = 0.003$) was observed. There was no significant influence of MTHFR C677T genotype on toxicity, OS and PFS.
Conclusions Leucovorin rescue given after high-dose MTX probably accounts for the lack of influence of C677T polymorphism. To better define a role of RFC1 polymorphism on patients outcome, it would be worthwhile to perform a study on intracellular MTX level and RFC1 substrate binding affinities in different genotypes.

Keywords High-dose methotrexate · MTHFR polymorphisms · RFC1 polymorphisms · Lymphoproliferative diseases · Toxicity · Outcome

Introduction

Methotrexate (MTX) is a key compound of chemotherapeutic regimens used in the treatment of acute lymphoblastic leukemia (ALL), primary central nervous system lymphoma (PCNSL) and Burkitt's lymphomas (BL). PCNSL is a rare condition representing about 5% of all central nervous system tumors. Standard treatment for PCNSL consists of high-dose MTX-based multi-agent chemotherapy followed by whole-brain radiation therapy for fit patients. In the elderly, chemotherapy alone is preferred due to its higher efficacy and reduced neurotoxicity compared to combined chemoradiotherapy [1].

BL is an aggressive form of non-Hodgkin B cell lymphoma due to a chromosomal translocation that involves the MYC oncogene [2]. The BL therapy consists of a high-intensity regimen based on the combination of cyclophosphamide, vincristine, doxorubicin, high-dose MTX and intrathecal therapy alternating with ifosfamide, etoposide, high-dose cytarabine and intrathecal therapy [3]. High-dose MTX regimens are also used in the treatment of ALL, especially in the presence of mature phenotype and in adolescents and young adults.

MTX is a chemotherapeutic agent that interrupts folic acid cycle: It acts by inhibiting two enzymes. Primarily, as analog of folate, MTX is a powerful competitive inhibitor

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of dihydrofolate reductase. Dihydrofolate reductase is responsible for converting folates to their active form tetrahydrofolate, which is a substrate of thymidylate synthase (TS). After that, the polyglutamated forms of MTX inhibit TS directly. After treatment with MTX, the cells will not be capable of "de novo" synthesis of purines and thymidylate. As a result, DNA synthesis will be inhibited. An important enzyme in the folate/MTX metabolism pathway is 5,10-methylenetetrahydrofolate reductase (MTHFR). MTHFR catalyzes conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in the folic acid cycle [4]. The MTHFR polymorphisms C677T and A1298C affect MTHFR enzyme causing a reduction in its activity, an altered distribution of intracellular folate metabolites and increased levels of homocysteine [5].

In the last years, a $G_{80}A$ polymorphism, which replaces His by Arg at position 27 of reduced folate carrier gene (RFC1), which encodes for the major MTX transporter, was identified [6]. The G variant correlated with lower plasma folate and higher homocysteine levels in healthy persons, and it was found at higher frequency in children with neural tube defects [7–10].

Because folate and homocysteine homeostasis are affected by MTX action, it is possible that these polymorphisms may also modulate the outcome in patients treated with this drug.

Our work is focused on the role of MTHFR C677T, MTHFR A1298C and RFC1 $G_{80}A$ polymorphisms on toxicity and outcome of patients affected by oncohematological disease requiring high-dose MTX therapy followed by leucovorin rescue.

Patients and methods

Between July 1994 and July 2009, we enrolled 54 patients, M/F: 29/25, with a median age of 52 years (range 15–78 years) affected by the following hematological diseases: 27 PCNSL, 15 BL, 10 ALL and 2 lymphoblastic lymphomas (Table 1).

All patients received high-dose MTX treatment, in particular 4 g/m^2 for patients affected by PCNSL and 1,200 mg/m^2 over 1 h and 240 mg/m^2 each subsequent hour for 23 h for patients affected by BL and ALL. Leucovorin was administered until serum MTX level was not detectable.

Peripheral blood samples were collected after obtaining informed consent from each patient or guardian for the use in biological studies. Every other day blood sampling was carried out.

MTX plasma concentrations were evaluated at 24 and 48 h from the start of the first dose of MTX infusion. Measurement of MTX serum levels was performed by immunoenzymatic assays.

Table 1 Patients' characteristics

Gender	
Male	29
Female	25
Median age (range)	52 years (15–78)
Disease	
LLA	10
Burkitt lymphoma	15
Lymphoblastic lymphoma	2
PCNSL	27
Toxicity	*N* (%)
Hematopoietic	38 (77%)
Grade I	3 (8%)
Grade II	10 (26%)
Grade III	2 (5%)
Grade IV	23 (61%)
Hepatic	25 (51%)
Grade I	10 (40%)
Grade II	9 (36%)
Grade III	6 (24%)
Renal	8 (16%)
Grade I	2 (25%)
Grade II	4 (50%)
Grade III	2 (25%)
Survival status	
Alive	25/49
Dead	24/49

DNA was extracted by Genomic DNA Isolation Kit (QIAamp DNA Blood).

Genotyping for the MTHFR C677T, A1298C and RFC1 $G_{80}A$ polymorphisms was performed using polymerase chain reaction/restriction fragment length polymorphism method as previously described [11, 12].

Toxicity was evaluated at day 7 after MTX administration. Organ toxicities were evaluated according to NCI-CTC criteria [13]. The following primary outcomes were evaluated: toxicity, relapse and survival rates according to the aforementioned polymorphisms.

Data were analyzed using SPSS 12.0 (SPSS. Chicago. IL-USA). χ^2 test was chosen for the analysis of the categorical factors. Kaplan–Meier method was applied to plot disease-free survival and overall survival curves. The Cox proportional hazard model was used to test the association of the covariates with outcomes of PFS and OS. Only variables those in univariate analysis reached a P value < 0.25 were included in the model. The stepwise procedure was used with backward Wald method. The results are expressed as hazard ratios (HRs) and 95% confidence intervals (CI95%). The significance level was set at $P < 0.05$.

Results

Out of 54 patients, only 49 were evaluable and entered the analysis. Six patients were not evaluable for the lack of complete clinical data.

The following prevalence of the different genotypes was observed: MTHFR C677T, 17 patients (35%) were wild type (CC), 18 (38%) had heterozygous genotype (CT), and 13 (27%) were homozygous (TT); MTHFR A1298C: 17 patients (35%) were normal (AA), 23 (48%) had heterozygous genotype (AC), and 8 (17%) were homozygous (CC); and RFC1 G80A, 13 patients (27%) were normal (GG), 18 (38%) had heterozygous genotype (GA), and 16 (34%) were homozygous (AA). The distribution of the three polymorphisms is quite similar to that reported in the Italian healthy controls from our previous study regarding MTHFR genotypes [14] and to the values reported in the study of Chango et al. about RFC1 polymorphisms [6], despite the large number in the control group (Table 2).

According to NCI-CTC criteria, we identified the following toxicities: 38/49 (77%) patients with hematological toxicity (grade I in 3 cases (8%), grade II in 10 cases (26%), grade III in 2 cases (5%) and grade IV in 23 cases (61%)); 25/49 (51%) patients with hepatotoxicity (grade I in 10 cases (40%), grade II in 9 cases (36%) and grade III in 6 cases (24%)); and 8/49 (16%) patients with renal toxicity (grade I in 2 cases (25%), grade II in 4 cases (50%) and grade III in 2 cases (25%)). Six out of 49 (12%) patients had elevated levels of homocysteine; 13/49 (26%) patients showed a folate deficiency, and 1/49 (2%) suffered a vitamin B12 deficiency prior to MTX administration (Table 1). Toxicity was analyzed after each HD-MTX administration.

During the observation time after high-dose MTX administration, thromboembolic episodes occurred in five patients (10%), including deep vein thrombosis in four cases and pulmonary embolism in one patient. This complication occurred only in PCNSL despite prophylaxis with low molecular weight heparin.

Overall survival (OS) and progression-free survival (PFS) rates for the whole group at 24 months were, respectively, 59 and 61%.

In univariate analysis of toxicity according to MTHFR genotypes, RFC1 $G_{80}A$ polymorphism and clinical characteristics including age, gender, plasma levels of homocysteine, folate, vitamin B12 and MTX, a significant association between hematological toxicity and age was found: Patients younger than 60 years of age experienced an increased toxicity (grade III–IV) compared to older patients (70% vs. 21%) ($P = 0.003$). MTHFR A1298C genotype only demonstrated that patients carrying MTHFR A1298C variant had a significant reduction in hematological ($P = 0.03$) and hepatic toxicity ($P = 0.02$) (Table 3).

There was no influence of RFC1 polymorphism and MTHFR genotypes on MTX plasma levels. Albeit not significant, we observed a trend between hematological toxicity and MTX plasma level ($P = 0.056$).

PFS rates were 54 and 38% at 1 and 2 years in RFC1 $G_{80}A$ wild-type patients and 80 and 68%, respectively, in heterozygous and homozygous patients: log rank test $P = 0.02$ (Fig. 1).

OS rates were 54 and 37% at 1 and 2 years in RFC1 $G_{80}A$ wild-type patients and 77 and 65%, respectively, in heterozygous and homozygous patients: log rank test $P = 0.035$ (Fig. 2).

In the multivariate model, including sex, age, RFC1 $G_{80}A$ and MTHFR A1298C polymorphisms and disease, RFC1 $G_{80}A$ polymorphism and age were the factors significantly associated with PFS: Older patients had a worse PFS [HR = 2.4 (95% CI 1,017–5,666) $P = 0.046$], and patients with RFC1 $G_{80}A$ variant had a better PFS [HR = 0.396 (95% CI 0,165–0,954) $P = 0.039$].

No difference was found using multivariate model for OS outcome.

Discussion

The main objective of this study was to determine how genetic variation in the folate pathway affects MTX toxicity and response in adult patients affected by hematological disease and treated with high dose of MTX.

Table 2 Genotype distribution of MTHFR 677, MTHFR 1298 and RFC1 polymorphisms in our patient group and in Caucasian population

Polymorphism	Patients			Caucasian healthy population		
MTHFR C677T	CC	CT	TT	CC	CT	TT
	17 (35%)	18 (38%)	13 (27%)	35 (31.8%)	55 (50%)	20 (18.2%)
MTHFR A1298C	AA	AC	CC	AA	AC	CC
	17 (35%)	23 (48%)	8 (17%)	56 (50.9%)	49 (44.5%)	5 (4.6%)
RFC1 G80A	GG	GA	AA	GG	GA	AA
	13 (27%)	18 (38%)	16 (34%)	46 (27.2%)	86 (50.9%)	37 (21.9%)

Table 3 Toxicities according to genotype frequencies

	MTHFR C677T *n* (%)				*P*	MTHFR A1298C *n* (%)			*P*	RFC1 G80A *n* (%)		*P*
	CC (%)	CT (%)	TT (%)	AA (%)		AC (%)	CC (%)	GG (%)		GA (%)	AA (%)	
Hematological toxicity	12 (31)	15 (40)	11 (29)	0.4	16 (42)	16 (42)	6 (16)	0.033	10 (26)	14 (37)	13 (34)	0.9
Hepatic toxicity	9 (36)	7 (28)	9 (36)	0.8	9 (36)	12 (48)	4 (16)	0.02	5 (20)	11 (44)	9 (36)	0.9
Renal toxicity	1 (12)	4 (50)	3 (38)	0.2	6 (75)	2 (25)	0	0.3	1 (12)	4 (50)	3 (38)	0.4

Influence of C677T and A1298C MTHFR polymorphisms on hematological malignancies has been extensively debated in the last years. In particular, the roles of both polymorphisms on the susceptibility to the ALL development, on the toxicity in course of chemotherapy with MTX and on the clinical response to chemotherapy have been largely discussed, and there are several and not univocal reports. Aplenc et al., in a population of 502 pediatric patients affected by ALL and treated with MTX, have shown that the C677T variant was statistically associated with disease relapse [15]. Krajinovic et al. have evaluated the influence of MTHFR polymorphisms A1298C, C677T and MTHFD1 1958 over EFS on 201 pediatric patients affected by ALL and receiving MTX chemotherapy, showing that T677A1298 haplotype and 1958A variant allele present a lower EFS [16]. Another study from Krajinovic et al. on 270 ALL patients and 300 healthy controls of French-Canadian origin demonstrated that CC677/AA1298 individuals developed more frequently ALL than individuals with different genotypes [17].

In a study of 100 ALL Korean patients, Kim et al. showed an increased risk of ALL development in patients with the MTHFR 677TT genotype [18]. In our previous study, we did not find any association between the MTHFR C677T and A1298C polymorphisms and susceptibility to ALL in a population of 174 patients and in 110 controls from central Italy [19].

Regarding the issue of toxicity during MTX therapy, some studies on 677C>T genotype showed increased toxicity in patients carrying the mutant T-allele as reported by Ongaro et al., who found an increased risk of hepatotoxicity, leukopenia and gastrointestinal toxicity [20], and by Lui et al. who confirmed these data on a Chinese population of 44 adult with ALL [21]. In another study on children affected by ALL and NHL, Kantar et al. suggested that subjects with MTHFR A1298C variant experienced increased hematological and hepatic toxicities [22]. Such data are confirmed in other clinical contexts: Toffoli et al. in a study about 42 female patients affected by ovarian cancer [23] and Urano in 106 patients affected by rheumatoid arthritis [24]. At the same time, other authors including Aplenc [15], Shimasaki [25] and Seidemann [26] did not find any correlation between MTHFR polymorphisms and development of toxicity.

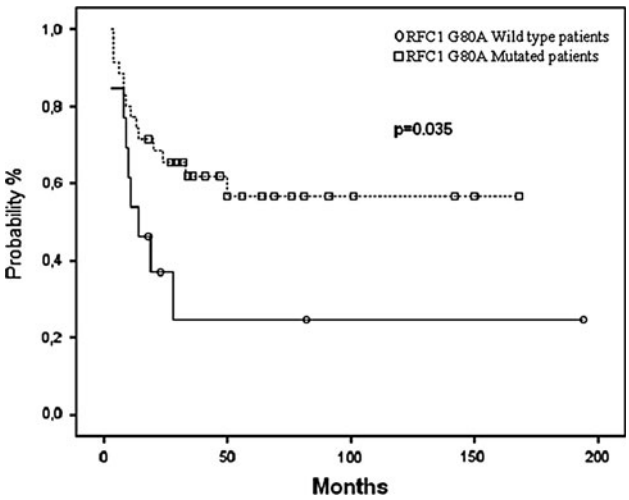

Fig. 1 Overall survival according to RFC1 genotype

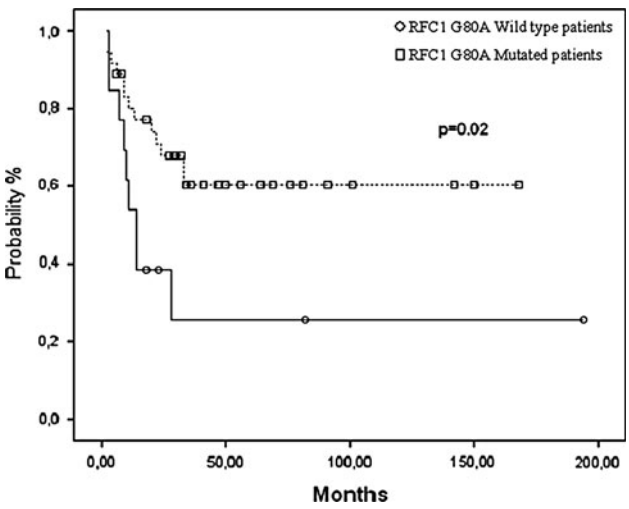

Fig. 2 Progression-free survival according to RFC1 genotype

In a previous study on a population of 82 adult patients with ALL treated during maintenance therapy with low MTX dosage, we observed that RFS and EFS between homozygous wild-type and variant patients in both MTHFR polymorphisms were not significantly different;

however, we found an association of the C677T variant with both survival and toxicity. These subset of ALL patients were homogenously treated with MTX-based maintenance in the absence of leucovorin rescue [27].

The present study identified no significant influence of MTHFR genotypes on toxicity, OS and PFS. In this setting, intensive leucovorin rescue takes place after high-dose MTX in order to reduce excessive toxicity deriving from prolonged drug plasma level abrogating the effect of MTHFR 677T variant observed when MTX is given at low doses for extended time without leucovorin administration. However, on univariate analysis, we found that patients with MTHFR 1298 AC/CC genotype were associated with a decreased hematological and hepatic toxicity. These data are in agreement with a previous study conducted by Huang et al.: The 1298A>C polymorphism results in a diminished enzyme activity; reduced conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate may lead to more substrate for TS and thereby to more DNA synthesis, which might result in lower incidence of side effects [28]. Furthermore, an "in vitro study" showed a decrease in MTX sensitivity of lymphoblasts obtained from pediatric patients with ALL and MTHFR 1298 variant [29].

Recently, another polymorphism was identified in one protein of the folate metabolic pathway: RFC1 is a major route for the transport of folate in mammalian cells, and it is an anion exchanger that transports folate as well as MTX into cells. The single nucleotide polymorphism 80G>A is associated with an Arg27His amino acid substitution, and this could interfere with folate and MTX transport into cells. In vitro studies have shown how the overexpression in human leukemia cells of a structurally altered RFC, which is characterized by a markedly increased affinity for folic acid and leucovorin, results in an expanded intracellular folate pool. Consequently, polyglutamylation of antifolate compounds is impaired, so their cytotoxic activity is abolished [30]. Otherwise, Whetstine et al. in an in vitro study showed no significant difference in MTX transport between the G and the A variant, whereas only a minor (twofold) difference in transport of 5′ formyl tetrahydrofolate cofactor was found [31]. The influence of RFC1 genotype on the folate and MTX pathway is controversial; investigations in patients with rheumatoid arthritis (RA) have shown that individuals with the RFC1 80AA genotype are more likely to have a superior clinical response to MTX therapy [32]. An increased intracellular content of MTX polyglutamates and consequently a major disease control has been supposed in RA. Chango et al. in a study on 169 healthy subjects explored the impact of this polymorphism, separately and in combination with C677T MTHFR genotypes, showing a moderate increase in total homocysteine levels in doubly homozygous 80GG/677TT subject; in addition, 80AA/677CT patients had higher plasma folate levels than the 80GG/677CT ones [6]. In our casistic, we found 6 patients both homozygous for 677TT and 80AA, but all of them showed normal homocysteine levels. On the other hand, other authors [25, 28, 33] demonstrated no significant differences in the development of toxicity or in the plasma MTX concentrations for the different RFC1 80G>A polymorphism.

Lavardière et al. in a study on 204 children affected by ALL showed an association between the genetic polymorphisms $G_{80}A$ and both MTX plasma levels and outcome; in fact, children with the A_{80} variant had worse prognoses and higher levels of MTX than the others genotype groups [34]. In contrast, Ashton et al. demonstrated that patients with RFC1 A_{80} variant showed a better EFS than wild type [35].

In our study, we found no influence of the RFC1 $G_{80}A$ polymorphism on toxicity development and no correlation with MTX plasma levels, but we point out a significant difference in OS and PFS rate according to RFC1 $G_{80}A$ genotype; in fact, in the Kaplan–Meyer analysis, patients carrying A_{80} variant had a better prognosis than the GG genotype, showing a better OS ($P = 0.035$) and PFS ($P = 0.02$) rates. These data are in agreement with Ashton et al. in their pediatric experience.

To better define a possible role of RFC1 polymorphism on patients outcome, it would be worthwhile to perform a study on intracellular MTX level and RFC1 substrate binding affinities in different genotypes.

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Conflict of interest None.

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