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Methotrexate pharmacogenetics in Uruguayan adults with hematological malignant diseases

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ABSTRACT

Background: Individual variability is among the causes of toxicity and interruption of treatment in acute lymphoblastic leukemia (ALL) and severe non-Hodgkin lymphoma (NHL) patients under protocols including Methotrexate (MTX): 2,4-diamino-N10-methyl propyl-glutamic acid.
Methods: 41 Uruguayan patients were recruited. Gene polymorphisms involved in MTX pathway were analyzed and their association with treatment toxicities and outcome was evaluated.
Results: Genotype distribution and allele frequency were determined for SLC19A1 $G_{80}A$, MTHFR $C_{677}T$ and $A_{1298}C$, TYMS 28 bp copy number variation, SLCO1B1 $T_{521}C$, DHFR $C_{-1610}G/T$, DHFR $C_{-680}A$, DHFR $A_{-317}G$ and DHFR 19 bp indel. Multivariate analysis showed that $DHFR_{-1610}G/T$ (OR = 0.107, p = 0.018) and $MTHFR_{677}T$ alleles (OR = 0.12, p = 0.026) had a strong protective effect against hematologic toxicity, while $DHFR_{-1610}CC$ genotype increased this toxicity (OR = 9, p = 0.045). No more associations were found.
Conclusions: The associations found between gene polymorphisms and toxicities in this small cohort are encouraging for a more extensive research to gain a better dose individualization in adult ALL and NHL patients. Besides, genotype distribution showed to be different from other populations, reinforcing the idea that genotype data from other populations should not be extrapolated to ours.

1. Introduction

Acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL) are within the annual 15,100 new cancer cases in Uruguay, according to the Comisión Honoraria de Lucha Contra el Cáncer. In the Uruguayan population, constituted by an estimate of 3,45 million people, the annual incidence reported for ALL is 1.76 and for NHL is 9.99 per 100,000, while the worldwide rate are 4.7 and 5.1 per 100,000 for leukemia and NHL respectively (Ferlay et al., 2015; Sociedad Uruguaya de Hematología, 2016).

For decades, Methotrexate (MTX): 2,4-diamino-N10-methyl propyl-glutamic acid, a structural analog of folic acid, has been commonly used in different types of cancer treatments (e.g. ALL, aggressive NHL, osteosarcoma, breast cancer, colorectal cancer, head and neck cancer), autoimmune diseases (e.g. rheumatoid arthritis, Crohn's disease, psoriasis) and rejection in hematopoietic cell transplantation. Due to its ability to inhibit enzymes involved in the cellular folate metabolism pathway, MTX leads to the inhibition of purine and pyrimidine precursors' synthesis, preventing DNA and RNA synthesis in rapidly dividing cells and causes defects in the methylation process (Chan and Cronstein, 2013).

MTX-induced toxicity is a matter of concern and can be the cause of treatment interruption or discontinuation, which may increase relapse risk. The primary toxicities of MTX affect the bone marrow and the gastrointestinal (GI) system. A bone marrow depression can occur abruptly leading to leucopenia, thrombocytopenia, anemia and megaloblastic anemia. Symptoms such as stomatitis, vomits, nausea, loss of appetite, diarrhea, oral and intestinal mucositis are indicators of GI toxicity. Stomatitis and diarrhea lead to treatment discontinuation, causing hemorrhagic enteritis, intestinal perforation or even death. Oral

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Table 1
Gene polymorphisms included in this work. Their interaction with MTX, most studied polymorphisms, their effect and previous studies references.

Gene	Interaction with MTX	Polymorphisms	Polymorphism effects	References
5,10-methylene tetrahydrofolatereductase (MTHFR)	Inhibition	$C_{677}T$ (rs1801133) $A_{1298}C$ (rs1801131)	Reduces enzyme activity	(Ongaro et al., 2009; Ranganathan et al., 2008; Spyridopoulou et al., 2012; Suthandiram et al., 2014)
Solute carrier family 19 member 1 (SLC19A1)	Uptake	$G_{80}A$ (rs1051266)	Reduces transporter affinity	(Dorababu et al., 2012; Faganel et al., 2011)
Dihydrofolatereductase (DHFR)	Inhibition either by MTX or MTX-PGs[a]	$C\text{-}_{1610}G/T$ (rs1650694) $C\text{-}_{680}A$ (rs442767) $A\text{-}_{317}G$ (rs408626) 19 bp indel[b] on intron 1 (rs70991108)	$C\text{-}_{1610}$, $A\text{-}_{680}$ and $G\text{-}_{317}$ alleles reduce enzyme levels. Del allele augments enzyme levels.	(Al-Shakfa et al., 2009; Askari and Krajinovic, 2010; Dulucq et al., 2008; Spyridopoulou et al., 2012)
Thymidylatesynthetase (TYMS)	Inhibition by MTX-PGs[a]	28 bp CNV[c] (rs34743033) of 2 (2R) or 3 (3R) repeats	3R allele augments enzyme level	(Lecomte et al., 2004; Ranganathan et al., 2008; Pakakasama et al., 2007)
Solute carrier organic anion transporter family member 1B1 (SLCO1B1)	Uptake	$T_{521}C$ (rs 4149056)	C allele reduces transport function and drug clearance	(Radtke et al., 2013; Ramsey et al., 2013; Relling and Ramsey, 2013; Zhang et al., 2014)

[a] MTX-PGs: active Methotrexate polyglutamate metabolite.
[b] Indel: insertion/deletion.
[c] CNV: copy number variation.

and intestinal mucositis are characterized by painful inflammation and ulceration of the mucous membranes lining the digestive system. During MTX chemotherapy, mucositis causes a significantly increase in infection and bleeding frequency, thereby reducing the therapy efficacy (Gibson et al., 2008; Ross et al., 2011). Acute or chronic liver damage, renal failure, central nervous system toxicity and skin reactions are less frequent adverse reactions (Gibson et al., 2008; Ross et al., 2011; Sweetman, 2009).

Germline genetic polymorphisms in genes encoding proteins that control the pharmacokinetics or pharmacodynamics of MTX, do not a priori affect disease predisposition, but may lead to variability in drug efficacy and safety in a small proportion of a population.

Several studies regarding MTX pharmacogenetics have reported genetic variations in candidate genes of the therapeutic drug pathway associated with toxicity and response (see references in Table 1). After a selection among several studies, the set of polymorphisms in candidate genes included in this work are listed in Table 1.

The study aimed to determine the genotype distribution of a group of gene polymorphisms involved in MTX pathway, as well as the influence on toxicity and treatment outcome, on Uruguayan adults with a hematological malignant disease under treatment with intermediate or high-dose MTX.

2. Patients and Methods

2.1. Patients and Study Design

A prospective longitudinal multicenter study was conducted on 41 Uruguayan adult patients diagnosed with hematological malignant disease (ALL or aggressive NHL), whose treatment included intermediate or high dose MTX. Doses were given according to the protocols HYPERCVAD-MA (1 g/m^2 intravenous (IV) + 12 mg intrathecal (IT) per even course and 20 mg/m^2 orally for 24 months during maintenance), Berlin-Frankfurt-Muensterwhich 2002 (5 g/m^2 IV during high risk induction and 8 g/m^2 IV during consolidation + 160 mg/m^2 orally during maintenance + 12–48 mg IT throughout protocol), CODOX-M-IVAC (3 g/m^2 IV + 12 mg IT per course) or MTX-ARAC (3.5 g/m^2 IV per course) (Ferreri et al., 2009; Mead et al., 2008; Stary et al., 2013; Thomas et al., 2004).

Exclusion criteria were hepatic or renal disease before starting treatment and not related to the hematological disease diagnosed.

A signed informed consent was obtained from all individual participants. The Ethical Committee of the School of Chemistry approved the study and is in accordance with the current version of the Helsinki Declaration.

2.2. Genotype Determination

From genomic DNA, obtained from peripheral blood, gene polymorphisms were evaluated employing different genotyping methods. Genotyping method conditions are described in Table 2.

In all cases, Sanger sequencing was used to confirm the results obtained by the different analysis methods employed.

2.3. Clinical Data

Patients were followed-up for clinical and laboratory parameters until they stopped receiving MTX treatment, in order to assess the development of therapy-related toxicity and clinical outcome (through remission and alive or deceased status). Data were collected 10 or 30 days after IV or oral administration, respectively.

GI (Nausea, vomiting, lack of appetite and oral ulcerations) and neurological symptoms, MTX dose modification, calcium folinate dose augment, remission, death, liver (Hepatic enzyme and bilirubin levels) and renal (Blood urea nitrogen and serum creatinine levels) functions, a complete blood count (Hemoglobin, leucocytes, neutrophils and thrombocytes) and the determination of MTX plasma levels were evaluated. Altered values were considered as a toxicity indicator.

The toxicity grading was recorded employing the Common Terminology Criteria for Adverse Events (CTCAE v4) scale. Nevertheless, this classification was not considered in our analysis, due to the low number of patients. Instead, each toxicity was analyzed in a dichotomous (presence or absence) form.

2.4. Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences software (SPSS V22.0, Chicago, IL, USA).

Univariate analysis was performed using Chi square test to estimate association between gene polymorphisms and clinical data of patients. The recessive and dominant models established the most frequent homozygous genotype as reference. A probability level (p value) was assumed significant if ≤ 0.05.

Multivariate analysis was performed using the logistic regression model for binary data, in which those variables with a p value ≤ 0.25 in the univariate analysis were included (Mickey and Greenland, 1989). Statistical significance was defined as p value ≤ 0.05.

Table 2
Genotyping method conditions for each genetic polymorphism studied.

Primer name	Primer sequence (5′-3′)	Annealing temp (°C)	$MgCl_2$ (mM)	DMSO 5%	Analysis method
SLC19A1F	CAGCGTCACCTTCGTCCCCTCC	54	1	+	PCR-RFLP
SLC19A1R	CAGGAGGTAGGGGGTGATGAAG				RE: *Hha*I
$DHFR_{19bpIndel}F$	ATGGGACCCAAACGGGCGCA	55	1	−	Real Time - HRM
$DHFR_{19bpIndel}R$	AAAGGGGAATCCAGTCGGGC				
DHFR	AGGAACAAAAGGGACCTTTCT	55	1.5	−	AS-PCR
DHFR	AATTCTCCAGAGGGCAGAAGAAG				
DHFR	AAGGAGAAAGTTGGGAGAAC				
DHFR	AATTCTCCAGAGGGCAGAAGATC				
DHFR	GGTCTATCACTGCCTACCTT				
DHFR	AATTCTCCAGAGGGCAGAAGACT				
DHFR	CTTCAATGCCCTTCCACATC	55	1.5	+	AS-PCR
DHFR	CAGAGATACTGCCACAGGAA				
DHFR	CTTCAATGCCCTTCCACATA				
DHFR	GATGTGACTAATCATGGAGTCC				
DHFR	ATTGAGCCATGCTGTTGCAG	WT: 58	1.5	+	AS-PCR
DHFR	CCTTGGTGGTCGAAGAGCT				
DHFR	CCACCCAAACTGTATTTCAGAC	M: 52			
DHFR	CCTTGGTGGTCGAAGAGCC				
$MTHFR_{677}F$	TGAGCCCAGCCACTCACTG	60	1.5	−	PCR-RFLP
$MTHFR_{677}R$	GTCGGTGCATGCCTTCAC				RE: *Hinf*I
$MTHFR_{1298}F$	AGAGCAAGTCCCCCAAGGA	55	1.5	−	PCR-RFLP
$MTHFR_{1298}R$	CTTTGTGACCATTCCGGTTTG				RE: *Mbo*II
$TYMS_{indel}F$	CGGGTTTCCTAAGACTCTCAGC	53	1.5	+	PCR
$TYMS_{indel}R$	AGCGGAGGATGTGTTGGATCTG				
SLCO1B1TF	TGGACTAATACACCATATTG	50	1.0	−	AS-PCR
SLCO1B1TR	AGCATATTACCCATGAAGA				
SLCO1B1CF	GGTCATACATGTGGATATATGC		1.5		
SLCO1B1CR	CAAAGGGAAAGTGATCATAC				

DMSO: Dimethyl sulfoxide added (+) or not (−). PCR: polymerase chain reaction. AS-PCR: allele specific PCR. PCR conditions were an initial denaturation of 3 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at annealing temperature, 30 s at 72 °C, with a final extension of 5 min at 72 °C. RFLP: restriction fragment length polymorphism. Enzyme digestion was performed at 37 °C for 3 h with 0.001 U of restriction enzyme (RE). PCR and digestion products were analyzed on 6% polyacrylamide gels. HRM: high resolution melting.

Table 3
Genotype distribution and allele frequency.

Genetic variants	Genotype distribution			Allele frequency	
	Homozygous wild type	Homozygous variant	Heterozygous	Wild type allele	Variant allele
SLC19A1 $G_{80}A$	26.8	26.8	46.4	0.50	0.50
DHFR 19 bp Indel*	29.3	24.4	46.3	0.52	0.48
DHFR C-$_{1610}$G/T	58.5	12.2	29.3	0.73	0.27
DHFR C-$_{680}$A	34.1	39.0	26.9	0.48	0.52
DHFR A-$_{317}$G	41.5	29.3	29.3	0.56	0.44
MTHFR $C_{677}T$	53.7	7.3	39.0	0.73	0.27
MTHFR $A_{1298}C$	41.5	7.3	51.2	0.67	0.33
TYMS 28 bp CNV(2R/3R)**	19.5	21.9	58.6	0.49	0.51
SLO1B1 $T_{521}C$	29.3	31.7	39.0	0.49	0.51

*Deleted and **3R alleles were considered as variant alleles.

3. Results

3.1. Demographic and Genetic Characteristics and Clinical Data

A total of 41 patients (20 ALL and 21 NHL, 29 males and 12 females) were included. The median age was 36.0 ± 13.9 in a range between 18 and 74 years.

Genotype distribution and allele frequency was calculated including the 41 patients and 55 healthy Uruguayan adults (31 males and 24 females, median age of 37.2 ± 12.5, range between 18 and 59 years) by direct counting (see Table 3).

Despite preventive measures, different kinds of toxicity in the patients group were observed: hematologic toxicity (regardless of the administration of calcium folinate), kidney toxicity (in spite of the adequate flow of alkaline urine maintained) and oral mucositis (despite mouthwash with borate, aluminum hydroxide gel, local anesthetics and antimycotic). Patient clinical data are listed in Table 4. Neurological toxicity was excluded from the statistical analysis because it did not present any variation among patients.

Table 4
Patient Clinical Data Toxicities evaluation were assessed as described in Method Section.

	Remission	Ca^{++} folinate dose augment	Hematologic toxicity	GI Toxicity	Liver toxicity	Kidney toxicity	Mucositis	MTX dose modification
Yes	20 (48.8%)	31 (75.6%)	26 (63.4%)	13 (31.7%)	16 (39%)	8 (19.5%)	7 (17.1%)	7 (17.1%)
No	21 (51.2%)	10 (24.4%)	15 (36.6%)	28 (68.3%)	25 (61%)	33 (80.5%)	34 (82.9%)	34 (82.9%)

Table 5
Univariate (A) and multivariate (B) analysis results.

A. Univariate analysis		
Hematologic toxicity	*p* value	OR (95% CI)
$MTHFR_{677}$ CC genotype (vs TT genotype)	0.026	4.500 (1.156–17.510)
$MTHFR_{677}$ CT genotype (vs CC genotype)	0.006	0.150 (0.037–0.614)
$MTHFR_{677}$ T allele (vs CC genotype)	0.047	0.265 (0.69–1.015)
DHFR-$_{1610}$ G/T alleles (vs CC genotype)	0.005	0.132 (0.029–0.594)
Liver toxicity		
DHFR-$_{680}$ CC genotype (vs AA genotype)	0.017	5.143 (1.279–20.677)
DHFR-$_{680}$ CA genotype (vs CC genotype)	0.017	0.100 (0.011–0.882)
DHFR-$_{680}$ A allele (vs CC genotype)	0.017	0.194 (0.048–0.782)
GI toxicity		
$MTHFR_{677}$ C allele (vs TT genotype)	0.017	0.094 (0.007–1.2)
MTX dose modification		
$SLCO19A1_{80}$ GG genotype (vs AA genotype)	0.047	5.143 (0.928–28.50)
$SLCO19A1_{80}$ A allele (vs GG genotype)	0.047	0.194 (0.035–1.078)

B. Multivariate analysis		
Hematologic toxicity	*p* value	OR (95% CI)
$MTHFR_{677}$ T allele	0.026	0.120 (0.010–0.7)
DHFR$_{-1610}$ G/T alleles	0.018	0.107 (0.015–0.950)
DHFR$_{-1610}$ CC genotype	0.045	9 (1.40–59)

OR: Odds ratio; CI: confidence interval; NA: not applicable when OR = infinity.

3.2. Association Studies Between Gene Polymorphisms and Clinical Data

In the univariate analysis a statistically significant association was found only with hematologic, liver and GI toxicity and MTX dose modification. No association was found with remission or death. Genotypes and alleles significantly associated (p value ≤ 0.05) are listed in Table 5A.

The univariate analysis showed associations between: hematologic toxicity and DHFR C-$_{1610}$G/T and MTHFR C_{677}T polymorphisms; liver toxicity and DHFR C-$_{680}$A polymorphism; GI toxicity and MTHFR C_{677}T, DHFR A-$_{317}$G and DHFR 19 bp indel polymorphisms; as well as MTX dose modification during therapy and SLC19A1 G_{80}A polymorphism.

Regarding hematologic toxicity, $MTHFR_{677}$ CC genotype (OR = 4.5, $p = 0.026$) increased the risk 4.5 times, while $MTHFR_{677}$ T allele (OR = 0.265, $p = 0.047$), $MTHFR_{677}$ CT genotype (OR = 0.150, $p = 0.006$) and DHFR-$_{1610}$ G/T alleles (OR = 0.132, $p = 0.005$) had a protective effect decreasing the risk 4, 7 and 8 times, respectively.

DHFR$_{-680}$ CC genotype (OR = 5.143, $p = 0.017$) increased the risk 5 times for liver toxicity, whereas DHFR$_{-680}$ A allele was seen to have a protective effect (OR = 0.194, $p = 0.017$).

GI toxicity was less frequent if $MTHFR_{677}$ C allele (OR = 0,094, $p = 0.032$) was present, given its protective effect decreasing the risk of occurrence 1 time.

A MTX dose modification was required along treatment in 17% of the patients and was 5 times more frequent in $SLC19A1_{80}$GG genotype patients (OR = 5.143, $p = 0.047$), while less frequent in $SLC19A1_{80}$ A allele carriers (OR = 0.194, $p = 0.047$).

On the basis of the results of univariate analysis (variables with a p value ≤ 0.25), the multivariate analysis shown that only hematologic toxicity and DHFR C-$_{1610}$G/T and MTHFR C_{677}T polymorphisms were significantly associated (p value ≤ 0.05).

DHFR-$_{1610}$ G/T alleles and $MTHFR_{677}$ T allele had a strong protective effect against hematologic toxicity, augmenting the risk 9 and 8 times, respectively, in patients without these alleles. In accordance with these results, DHFR-$_{1610}$ CC genotype increased the risk of suffering this kind of toxicity 9 times in comparison with non-carriers (see Table 5B).

Lastly, we could not replicate previously reported associations between 28 bp CNV TYMS or SLCO1B1 T_{521}C and treatment response (Lecomte et al., 2004; Pakakasama et al., 2007; Radtke et al., 2013; Ramsey et al., 2013; Ranganathan et al., 2008; Relling and Ramsey, 2013; Zhang et al., 2014;).

4. Discussion

We were interested in replicate in Uruguay previous pharmacogenetic studies performed abroad, in order to gain knowledge of a population with a particular ethnicity (84.1% European; 10.4% Amerindian; 5.6% African) (Sans, 2009).

The results of the multivariate analysis showed that DHFR-$_{1610}$ G/T alleles and $MTHFR_{677}$ T allele had a protective effect against hematologic toxicity, while DHFR-$_{1610}$ CC genotype carriers had an increase risk.

These results are expected in the context of MTX treatment due to the involvement of these enzymes in DNA synthesis, resulting in damage to the blood cells. MTX and its active polyglutamated forms competitively and indirectly inhibit the DHFR and MTHFR enzymes (Chen and Shen, 2015). This inhibition results in blockage in the de novo purine and pyrimidine synthesis, causing impaired DNA formation, DNA methylation and gene regulation.

The role of DHFR C-$_{1610}$G/T polymorphism in MTX pharmacogenetics has been poorly studied. Dulucq et al. (2008) reported that DHFR-$_{1610}$ CC genotype carriers presented a 21.4% increase in DHFR mRNA levels leading to a 1.7 times higher risk of suffering low event free survival ($p = 0.02$).

Regarding the association between MTHFR C_{677}T polymorphism and hematologic toxicity, a large number of studies have been performed and reported conflicting results. Some disagreed with our results, reporting that TT genotype and/or T allele are responsible for MTX-induced toxicity (Zhao et al., 2016; Eissa & Ahmed, 2013; Zgheib et al., 2014; Ongaro et al., 2009; Kitamura et al., 2008) or did not find any kind of association (Campbell et al., 2016; Chiusolo et al., 2012; Erčulj et al., 2012). Jabeen et al. (2015) even found that $MTHFR_{677}$C allele was associated with higher degree of liver toxicity (88%, $p = 0.007$).

In consequence, the results of association studies between gene polymorphisms and treatment response differ widely, depending on the patient groups, their ethnicity, treatment protocols, or the statistical models employed. Full knowledge concerning folate pathway gene variants and their association with toxicity and outcome in MTX treatment is still part of an unfinished topic. Further studies with larger patient number are required to validate our findings, although extensive studies with large number of patients also yield contradictory results.

In this first MTX pharmacogenetic study performed in Uruguay, the distribution of SLC19A1 $G_{80}A$, MTHFR $C_{677}T$, TYMS 28 bp copy number variation, SLCO1B1 $T_{521}C$, DHFR $C_{-1610}G/T$ and DHFR$C_{-680}A$ genotypes differed from those available on SNP database (dbSNP NCBI, 2017) and previous reports (González-Mercado et al., 2014; Hagleitner et al., 2014; Zgheib et al., 2014). These differences reinforce the idea that available genotyping data from other populations should not be extrapolated to ours.

Although more extensive genetic molecular studies must be performed, the results in this small cohort of Uruguayan patients with a particular ethnicity are encouraging.

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Conflict of Interest

The authors have no conflicts of interest.

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