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G80A Single Nucleotide Polymorphism in Reduced Folate Carrier-1 Gene in a Mexican Population and its Impact on Survival in Patients with Acute Lymphoblastic Leukemia

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ABSTRACT

Background: Hyper-CVAD is the treatment for patients with acute lymphoblastic leukemia in our institution. **Objective:** To evaluate the impact of single nucleotide polymorphisms at genes associated with methotrexate metabolism on survival. **Methods:** The presence of the single nucleotide polymorphisms G80A at *reduced folate carrier-1* gene and C677T in the *methylenetetrahydrofolate reductase* gene was determined by denaturing high performance liquid chromatography and validated by sequencing. Both single nucleotide polymorphisms were evaluated in 71 healthy donors and in an exploratory pilot trial with acute lymphoblastic leukemia patients to determine the influence of these single nucleotide polymorphisms on clinical outcome. Clinical characteristics, response, and outcome were registered. A Cox regression analysis was done to evaluate factors influencing response and overall survival. **Results:** There were no differences in the frequency of single nucleotide polymorphisms between volunteers and acute lymphoblastic leukemia patients according to the Hardy-Weinberg test. Sensitivity and specificity were 72 and 91% for the G80A, and 64 and 75% for the C677T, respectively. The multivariate analysis showed that the T-immunophenotype and the presence of single nucleotide polymorphism G80A *reduced folate carrier-1* were associated with a shorter relapse-free survival and overall survival. **Conclusions:** The presence of G80A single nucleotide polymorphism at reduced folate carrier-1 gene in acute lymphoblastic leukemia patients was associated with a poorer prognosis. (REV INVES CLIN. 2016;68:154-62)

Key words: RFC1 gene. MTHFR4 gene. SNP. Leukemia. Methotrexate.

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) in adults remains a significant treatment challenge, in contrast with pediatric ALL where considerable improvements in long-term survival and even cure have been achieved over the last 30 years. Long-term overall survival for this group remains relatively poor, between 20 and 40%. Current research in adult ALL has mainly focused in optimizing the use of cytotoxic drugs. In this regard, pharmacogenetics is of considerable interest, particularly for methotrexate (MTX), a folate analog drug that is essential in regimens for ALL[1-5].

The influx of MTX into cells depends on the reduced folate carrier protein (RFC), which efficiently transports folates and MTX into cells and, once inside, is converted into methotrexate polyglutamates (MTXPG) by the enzyme folyl-polyglutamate synthetase[6,7]. The MTX and its polyglutamates affect intracellular folate metabolism by inhibiting dihydrofolate reductase and thymidylate synthase. Therefore, the inhibition of DNA biosynthesis induced by MTX is multifactorial, including both partial depletion of reduced folates and direct inhibition of folate-dependent enzymes. The effectiveness of MTX depends on its concentration and retention in cells.

The *RFC1* gene is located on the long arm of chromosome 21 (21q22.2-22.3) and encodes a membrane protein called reduced folate carrier[8]. The RFC1 single nucleotide polymorphism (SNP) 80G>A (rs1051266) leads to the substitution of guanine for adenine in the 80th position, which results in the substitution of arginine for histidine at the residue 27 in the structure of the protein[9]. Chan, et al. showed that this change results in decreased receptor affinity and variations in the transmembrane transport of folic acid antimetabolites. In *ex vivo* studies, the folate concentrations in serum were higher in the 80AA genotype than the allele G variant: 19 vs. 15 mmol/l, respectively[10]. Banerjee, et al. analyzed the relationship between the *RFC1* G80A polymorphism and the risk of relapse of ALL in children. They found that in 204 ALL patients studied, the *RFC1* 80AA variant was associated with higher serum concentrations of MTX[11], which has also been observed by other authors[12].

On the other hand, the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate in the folic acid cycle[12]. A common genetic polymorphism in the *MTHFR* gene results from a C®T substitution. Individuals with the T/T genotype commonly have elevations in plasmatic homocysteine and differences in response to folic acid supplementation compared with normal (C/C) or heterozygous (C/T) genotypes. This polymorphism is highly prevalent in the Mexican population[13-15], particularly among Nahua and Mixtec groups. However, in other regions of the country, such as the north, the prevalence is similar to Caucasian regions[14]. In addition, this SNP may influence the therapeutic response to antifolate drugs such as MTX. The frequencies of the C677T allelic variants differ according to ethnicity. In Europe, 8-20% of the Caucasian population is homozygous for the 677T allele and almost 40% is heterozygous[2,16]. Although some authors[12,17] have described the influence of this SNP on survival and toxicity in patients with ALL whose treatment includes MTX, its role is still unclear. Thus, in this work we evaluated the feasibility of using denaturing high-performance liquid chromatography (DHPLC) to determine *RFC1* and *MTHFR* gene polymorphisms as well as to correlate these SNPs with the toxicity and outcome in adults with ALL receiving MTX as part of the hyper-CVAD regimen.

MATERIAL AND METHODS

Study population

Patients

A total of 31 adult patients with ALL were included from January 2011 to December 2012 at the National Cancer Institute (INCan) in Mexico in this prospective, exploratory pilot trial to assess the influence of the *RFC1 G80A and MTHFR C677T* SNPs on response and overall survival.

Healthy individuals

Blood samples from 71 consecutive healthy, volunteer blood donors were obtained by venous puncture from the arm; none of the donors were related to our ALL patients.

Inclusion criteria

The patients were untreated, Mexican, older than 15 years of age, and with normal renal and hepatic function. After inclusion and blood sample collection, patients began treatment with hyper-CVAD. Patients with a history of hypersensitivity to MTX or to any of the other drugs included in the hyper-CVAD regimen were excluded. This protocol was approved by the IRB Committee and registered at www.clinicaltrials.gov (Identifier # NCT01307241). Healthy individuals and patients signed an informed consent.

Baseline clinical and pathological characteristics were recorded. Patients were assessed for response using the International Working Group Criteria for acute leukemia[18]. Overall survival (OS) was defined as the time since diagnosis until death or date of the last visit. Relapse-free survival (RFS) was defined as the time since remission was achieved until relapse was documented.

Laboratory procedures

DNA extraction

Genomic DNA was obtained using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The DNA was quantified in a NanoDrop (Applied Biosystems) and stored at −20 °C.

Polymerase chain reaction amplification

Polymerase chain reaction (PCR) was performed using the following oligonucleotides: Forward: 5'-AGT GTCACCTTCGTCCC-3' Reverse: 5'-TCCCGCGTGAAG TTCTTG-3' for the *RFC1* gene, and Forward: 5'- GG AGCTTTGAGGCTGACCTGAA-3' Reverse: 5'-AGGAC GGTGCGGTGAGAGTG-3' for the *MTHFR* gene. The PCR was performed in a total volume of 25 µl containing 100 ng genomic DNA, 1 µmol/l oligonucleotides (forward and reverse), 200 µM dNTPs (Fermentas Life Sciences, USA), 0.25 U optimase polymerase enzyme (Applied Biosystems), and PCR 1 x buffer (15 mM MgCl2, Perkin Elmer, Foster City, CA). The PCR was performed on a GeneAmp® 9700 thermal cycler (Applied Biosystems) using an initial denaturation step at 94 °C for five minutes, followed by 40 cycles at 94 °C for 30 seconds, annealing (60 °C for *RFC1* and 55 °C for *MTHFR*) for 30 seconds, and 72 °C for 30 seconds; a final extension was performed at 72 °C for seven minutes. Products were electrophoresed in 2% agarose gels.

Denaturing high-performance liquid chromatography analysis

The PCR products were denatured at 95 °C during 10 minutes and were cooled until 25 °C, decreasing 2 °C/minute to allow for homo- or heteroduplex formation (Transgenomics, Inc; San José, CA) according to conditions determined by DHPLC software. Heterozygous chromatograms were identified by visual analysis and compared with the wild type. Homoduplex cases, which have a single peak chromatogram as wild type, were mixed with known wild type DNA (previously sequenced), to allow heteroduplex formation. Results were reported as wild type (wt) or polymorphic.

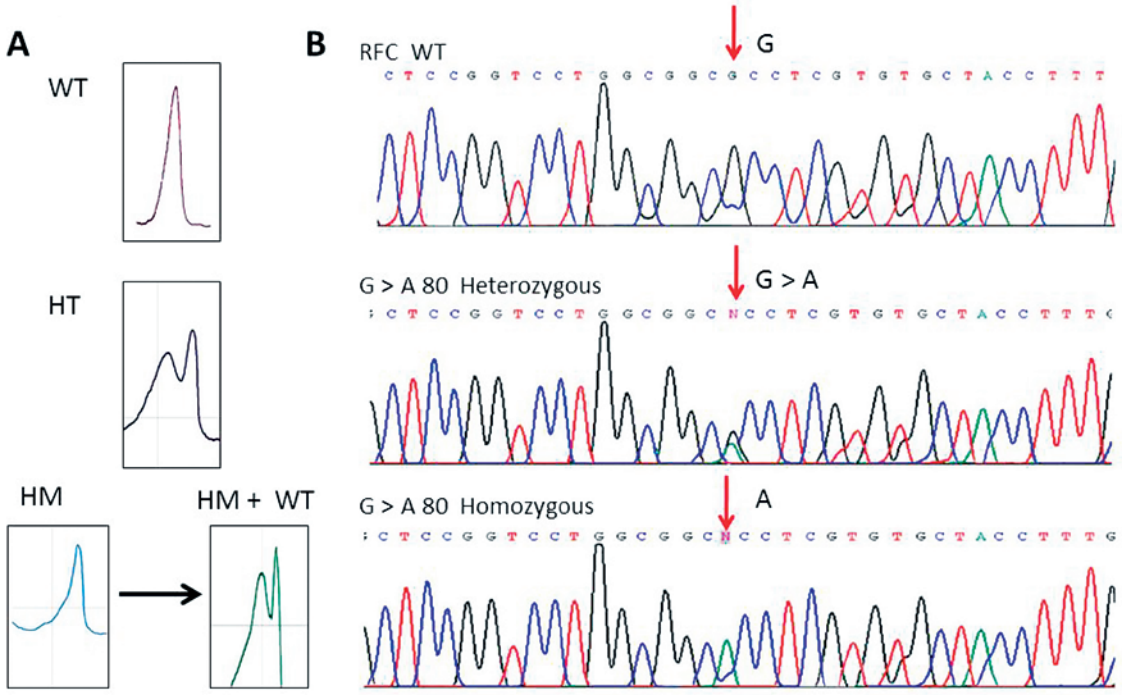
Sequencing

Seventy-one samples from healthy individuals were sequenced (gold standard), regardless of the results of DHPLC analysis from at least two independent amplification products. The ALL samples were amplified and analyzed by DHPLC. The PCR amplicons were purified using isopropanol precipitation, diluted and cycle-sequenced using a BigDye® Terminator kit v3.1 (ABI, Foster City, CA) according to manufacturer instructions in an ABI Prism® 3100 genetic analyzer. Electropherograms were analyzed in both sense and antisense directions.

Statistical analysis

The SNP frequencies were compared between healthy individuals and patients using the Hardy-Weinberg test. The results of DHPLC and Sanger sequencing (gold standard) were analyzed to determine sensitivity: (true positive/ [true positive + false negative]), and specificity: (true negative/ [false positive + true negative]). Survival curves were done by Kaplan-Meier method and compared by log rank test. Cox regression analysis was done to evaluate factors influencing response and overall survival.

Figure 1. RFC gene analysis by **A:** DHPLC showing a single peak for the homozygous wildtype or mutant, whereas multiple peaks were indicative of heteroduplexes containing G>A 80 SNP. When an homozygous sample mixed with wildtype DNA showed a multiple peak profile, indicated the presence of the SNP homozygously. **B:** Sanger sequence confirming DHPLC finding.



RESULTS

Healthy individuals

Blood samples were collected from 71 healthy donors. The mean age was 30.6 years (range 19-57) and 48% were male.

Sensitivity and specificity of DHPLC in healthy individuals: PCR fragments of *MTHFR* and *RFC*1 genes were subjected to DHPLC, and the conditions for analysis (gradient and temperature) were optimized for each fragment to yield characteristic and reproducible profiles. As shown in figure 1, samples with *wt RFC1* sequence eluted as a single peak, whereas multiple peaks were indicative of heteroduplex containing 80G>A SNP. Homozygous polymorphic also showed a single peak, and an elution mixed with a *wt* sample was required to demonstrate this status. These results were confirmed by Sanger sequencing. Likewise, *MTHFR wt* showed a single peak and C>T 677 SNP was documented with multiple peaks (Fig. 2). As shown in table 1, regarding the *MTHFR* SNP from the 71 samples from healthy individuals, 38 showed a polymorphic chromatogram, which was confirmed by sequencing in 33 (true positive); five cases were recorded as false positive (polymorphic chromatogram by DHPLC, but the SNP discarded by sequencing). Eighteen cases had a *wt* chromatogram, but sequencing showed the SNP (false negative), whereas 15 were true negative (*wt* chromatogram and no SNP by sequencing). The calculated sensitivity and specificity were 64 and 75%, respectively, for *MTHFR*, and 72 and 91% for the *RFC1* gene, respectively (Table 1).

Patients

The clinical and pathological characteristics of ALL patients are shown in table 2. The mean age was 26 years (range 16-64); male:female distribution was 17:14. Most (74%) had the common B subtype and six (20%) were positive for the Philadelphia chromosome.

After analyzing DHPLC chromatograms for these polymorphisms, the frequency of the *RFC1* G80A was 50.7% in healthy individuals and 40.7% among leukemia patients. All were heterozygous. Regarding the *MTHFR* C677T SNP, the frequency was 52.7 and

Figure 2. MTHFR gene analysis by **A:** DHPLC showing a single peak for the homozygous wildtype or mutant, whereas multiple peaks were indicative of heteroduplexes containing C > T677 SNP. When an homozygous sample mixed with wildtype DNA showed a multiple peak profile, indicated the presence of the SNP homozygously. **B:** Sanger sequence confirming DHPLC finding.

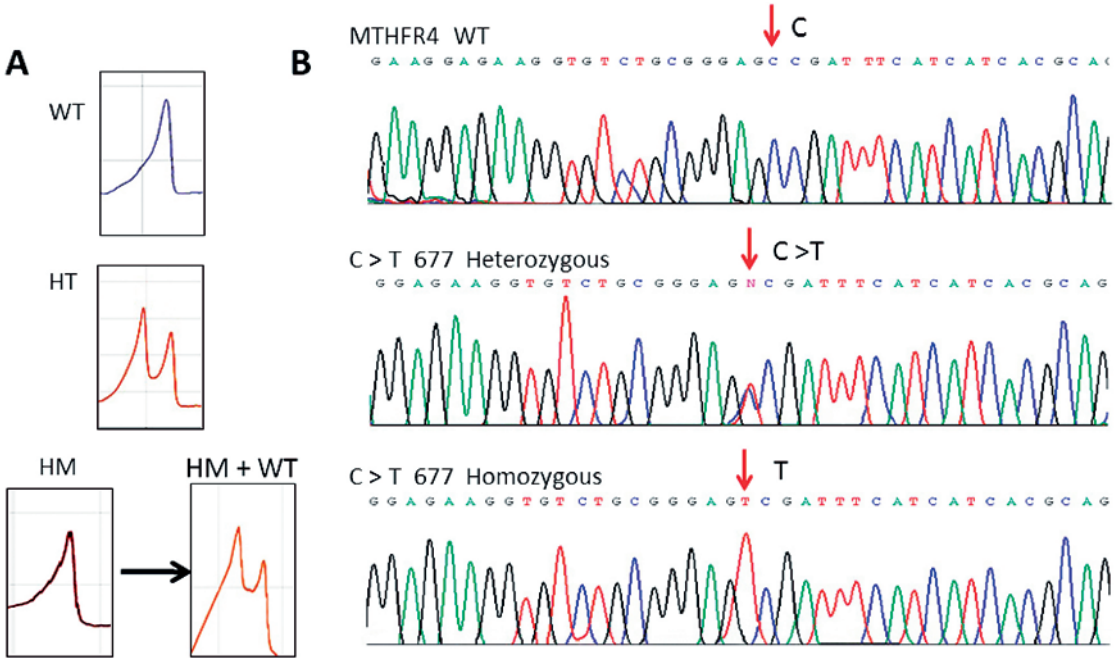


Table 1. Sensitivity and specificity of the denaturing high performance liquid chromatography technique for *methylenetetrahydrofolate reductase* and *reduced folate carrier-1* genes in 71 healthy blood donors

	MTHFR	*RFC1*
True positive (n)	33	34
False positive (n)	5	2
False negative (n)	18	13
True negative (n)	15	22
Total (n)	71	71
Sensitivity (%)	64	72
Specificity (%)	75	91

MTHFR: methylenetetrahydrofolate reductase; RFC: reduced folate carrier.

Table 2. Clinical characteristics of acute lymphoblastic leukemia patients

	Patients
	n = 31
Mean age, years (range)	26 (16-64)
Male:female	17:14
ALL classification (n)	
Pre-B	2
Pro-B	4
Common B	23
Mature B	1
T	1
Cytogenetic analysis (n)	
Philadelphia positive	6
Normal	25
CNS infiltration at diagnosis, n (%)	3 (9.6)

ALL: acute lymphoblastic leukemia; CNS: central nervous system.

72.4%, respectively. There was only one homozygous patient in the leukemia population. After using Hardy-Weinberg test, no statistically significant difference was found with that expected for the analyzed population.

Toxicity

As expected, all patients had grade 4 myelosuppression. No grade 3-4 liver toxicities were documented, and there were no differences in toxicity rates between patients having wild type or any of the SNPs.

In accordance with the international standard criteria, complete response was achieved with the hyper-CVAD regimen in 25 cases (80.6%), and partial response in the remaining six cases. The three-year RFS and OS were 42 and 22%, respectively. Median RFS was

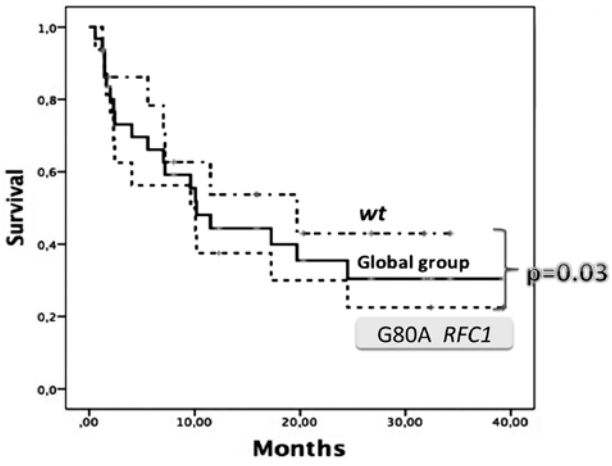
Table 3. Cox regression analysis of factors influencing overall survival and relapse-free survival in acute lymphoblastic leukemia patients

Variable	Overall survival			Relapse-free survival		
	HR	95% confidence interval	p value	HR	95% confidence interval	p value
Gender	1.43	0.88-7.30	0.083	2.6	1.1-205.8	0.930
Age	1.1	1.06-1.94	0.100	1.5	0.56-1.69	0.940
MTHFR C677T SNP	0.68	0.42-1.40	0.183	0.74	0.52-1.10	0.379
RFC1 G80A SNP	1.5	1.2-1.7	0.039	3.8	1.39-10.00	0.0463
Philadelphia chromosome	1.8	0.4-7.0	0.483	0.9	0.11-7.20	0.426
Immunophenotype	1.9	1.2-3.7	0.008	1.1	0.32-2.70	0.039

MTHFR: methylenetetrahydrofolate reductase; RFC: reduced folate carrier; SNP: single nucleotide polymorphism.

1.3 years (95% CI: 0.92-1.8). Median OS was 1.7 years (95% CI: 1.4-2.6). Cox regression analysis was done to evaluate prognostic factors, including age, cytogenetic analysis, immunophenotype of ALL, and presence or absence of any of the SNPs. The presence of 80*G>A RFC* SNP and T-cell immunophenotype was associated with decreased RFS and OS (Table 3 and Fig. 3).

Figure 3. Overall Surviva.



DISCUSSION

The pharmacogenetic approach in chemotherapy constitutes a research field developed to optimize drug doses and schedules for either increasing response rate or reducing toxicity. Hyper-CVAD is considered a standard of care for patients with ALL in many centers worldwide, including our institution[19]. Within this schema, methotrexate constitutes a key drug for ALL treatment. The results of this work add information on the field of pharmacogenetic regarding the impact of the *RFC1* 80G>A SNP on the efficacy of MTX when used in the context of hyper-CVAD for adult ALL patients. Although many other factors related to clinicopathological characteristics of ALL patients were not taken into account, as well as other pharmacogenetic factors that may modulate the effect of the drugs other than MTX in the hyper-CVAD regimen, Cox regression analysis clearly suggest that the *RFC1* 80G>A SNP may help to optimize the dose of MTX when used within this regimen, although this should be confirmed in further studies that include a larger sample of patients.

The *RFC* 80G>A polymorphism has been widely studied in diverse populations searching for associations with increased leukemia risk and with the clinical course (toxicity and prognosis) in leukemia patients receiving MTX. Data from the 71 healthy individuals in our study suggest that our populations have an increased frequency of this SNP (50.7%), similar to that reported among healthy French individuals (36%), whereas in a British population the frequency was 41%[5]. A higher frequency (71%) has been found in a Jordanian female population with rheumatoid arthritis[20]. In this respect, some authors[5] have suggested a relationship between the *RFC1* 80G>A polymorphism and a 2.1-fold increased risk of ALL. Furthermore, Huang, et al.[21], after a stratified analysis by ethnicity, recently demonstrated that the association became more prominent among Caucasians (GA vs. GG: OR: 1.28; 95% CI: 1.12-1.45; $p < 0.001$). In contrast with these results, our findings do not support this association, since there was a trend for a higher frequency of the *RFC1* 80G>A polymorphism in the healthy population compared with ALL patients (50.7 vs. 40.7%, respectively).

Regarding the association between this polymorphism and shorter RFS and OS, there are experimental data supporting it, although there is also contradictory information.

In contrast to our results, in a study including 70 children with ALL, an association was found between the polymorphism and a lower risk of relapse ($p < 0.05$): in patients with the G/A genotype it was 3.97 (95% CI: 1.12-14.06) compared with carriers of the A/A genotype (*wt*) who had a higher probability of relapse (7.84; 95% CI: 1.66-37.10)[22]. In our patients, those with the G/A genotype had a 3.8 HR for relapse.

Other authors[23,24] found that extra copies of chromosome 21, where the *RFC1* gene is located, were associated with increased expression of mRNA and reflect elevated capacities for MTX transport. Our findings are supported by a decreased functional status of this SNP, showing a lower intracellular folate concentration in patients with the *RFC* 80G>A polymorphism, as has been confirmed by Yates and Lucock[25], although other manifestations of dysfunction of this 80G>A polymorphism have not been documented, such as a higher risk of neural tube defects or colorectal cancer[25-29]. In this regard, Chan, et al. showed that this change results in decreased receptor affinity and variations in the transmembrane transport of folic acid antimetabolites. In *ex vivo* studies, the folate concentrations in serum were higher in the 80AA genotype than the allele G variant: 19 vs. 15 mmol/l, respectively[10]. Banerjee, et al. analyzed the relationship between the *RFC1* G80A polymorphism and the risk of relapse in 204 children with ALL and also found that the *RFC1* 80AA variant was associated with a higher serum concentration of MTX[11], which has also been observed by other authors[12]. However, Whetstine, et al.[30] reported that the change of the strongly basic amino acid arginine to histidine, which is a weak base, in a region of the carrier documented to influence folate substrate binding and rates of uptake, might be expected to alter *RFC* transport properties. Nevertheless, by directly comparing the transport properties of Arg27 and His27-hRFC in stable K562 transfectants, no significant differences in the uptake rates of MTX were observed, and only minor differences were calculated in the relative affinities for an assortment of transport substrates. Collectively, these data strongly argue for a lack of major functional differences between the Arg27- and His27-RFCs for reduced folate cofactors and for various antifolates used in cancer chemotherapy, including MTX. On the other hand, researchers in Japan have shown increased liver toxicity in homozygous 80AA, and increased side effects in the form of severe vomiting in the 80GG group homozygotes[31,32]. We found no differences regarding toxicity dependent on this SNP, which may be explained because we had only one homozygous patient (80GG).

In contrast with the *RFC1* 80G>A polymorphism, in our study, the SNP 677C>T at the *MTHFR* gene did not influence RFS or OS. Similar results have been obtained by other authors. A multicenter study from Poland published in 2006[32], in which *MTHFR, TPMT, GSTT1, GSTM1, GSTP1,* and *TS* polymorphisms were determined, showed a significant relationship between genotype 677C>T and an increased death rate (OR: 4.09; 95% CI; $p = 0.028$). Eight of the 31 (26%) patients whose death occurred during treatment had homozygous 677TT, but there was no association with the genotype 677C>T. In line with our results, Deus, et al.[33] also reported that *G80A* polymorphism influenced the survival of pediatric patients with ALL, but neither *G677T* nor *A1298C* in *MTHFR* gene had an effect on survival. In Mexico, the presence of *MTHFR* has been evaluated in different regions of our country[13-15,34] and strong differences in frequencies of C677T polymorphism were documented, being higher among Nahua and Mixtec groups compared with Mestizos[13,14]. Additionally, Ruiz-Argüelles, et al.[34] evaluated the risk of mucosal damage in 28 patients with ALL treated with MTX and, in accord with our findings, they concluded that there was no significant association with mucositis at the gene or at the genotype level. They also postulated that the risk of higher MTX toxicity in patients with decreased MTHFR activity could be neutralized by the normally folate-rich diet in Mexico.

Results of our study demonstrate by multivariate analysis that the G80A SNP is associated with shorter RFS and OS. However, because of the small sample size and unknown clinical data, it is possible that results were due to random effects. In addition, there are more pharmacogenetic variations of genes implicated not only in the metabolism of MTX, but of the other drugs in the hyper-CVAD regimen that were not studied. Therefore, it is possible that their interactions could explain the results observed.

Nevertheless, our results regarding the influence of the G80A polymorphism cannot be underestimated since other studies[1,5,7] have also found this association in leukemia patients receiving MTX. Further studies are needed to establish the value of this pharmacogenetic marker in the optimization of leukemia treatment with MTX.

Finally, our study to determine the feasibility of using DHPLC as a routine method to determine the SNPs here studied and others[35-37], suggest that it can be of value, despite the fact that the sensitivity and specificity we obtained were not so high. However, a number of papers have documented the excellent sensitivity and specificity of DHPLC in detecting mutations[38]. For instance, O'Donovan, et al. have reported a sensitivity and specificity of 100% for detecting mutations in exon H of the Factor IX and exon 16 of the neurofibromatosis type 1 gene[39]. However, under a single hybridization condition, some probes do not have optimal hybridization kinetics and therefore markers located near such sequence contexts cannot be detected. In addition, it is difficult to identify markers that are present as heterozygotes, as well as markers located close to other polymorphisms. As a result, most studies reach a sensitivity of 85-95%, with specificity in some cases as low as 55%[40]. In this regard, the relatively lower sensitivity and specificity here found can potentially be increased by testing different conditions. Regarding the polymorphisms studied, these are preliminary results and require confirmation. However, we could suggest that patients with G80A may be treated with other regimens without methotrexate, or within clinical trials.

REFERENCES

1. Mei L, Ontiveros EP, Griffiths EA, Thompson JE, Wang ES, Wetzler M. Pharmacogenetics predictive of response and toxicity in acute lymphoblastic leukemia therapy. Blood Rev. 2015; 29:243-9.
2. Candelaria M, Taja-Chayeb L, Arce-Salinas C, Vidal-Millan S, Serrano-Olvera A, Dueñas-Gonzalez A. Genetic determinants of cancer drug efficacy and toxicity. Practical considerations and perspectives. Anticancer Drugs. 2005;16:923-33.
3. Hooijberg JH, Broxterman HJ, Kool M, et al. Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Res. 1999;59:2532-5.
4. Kool M, van der Linden M, de Haas M, et al. MRP3, an organic anion transporter able to transport anti-cancer drugs. Proc Natl Acad Sci USA. 1999;96:6914-19.
5. Hider I, Bruce N, Thomson W. The pharmacogenetics of methotrexate. Rheumatology. 2007;46:1520-4.
6. Payne K, Newman W, Fargher E, Tricker K, Bruce IN, Ollier WE. TPMT testing in rheumatology: any better than routine monitoring? Rheumatology. 2007;46:727-9.
7. Pirmohamed M. Pharmacogenetics and pharmacogenomics. Br J Clin Pharmacology. 2001;52:345-7.
8. Genestier L, Paillot R, Quemeneur L, Izeradjene K, Revillard JP. Mechanisms of action of methotrexate. Immunopharmacology. 2000;47:247-57.
9. Stanisławska-Sachadyn A, Mitchell LE, Woodside JV, et al. The reduced folate carrier (*SLC19A1*) c.80G>A polymorphism is associated with red cell folate concentrations among women. Ann Hum Genet. 2009;73:484-91.
10. Chan ES, Cronstein BN. Molecular action of methotrexate in inflammatory diseases. Arthritis Res. 2002;4:266-73.
11. Banerjee D, Mayer-Kuckuk P, Capiaux G, Budak-Alpdogan T, Gorlick R, Bertino JR. Novel aspects of resistance to drugs targeted to dihydrofolate reductase and thymidylate synthase. Biochim Biophys Act. 2002;1587:164-73.
12. de Deus DM, de Lima EL, Seabra Silva RM, Leite EP, Cartaxo Muniz MT. Influence of methylenetetrahydrofolate reductase C677T, A1298C, and G80A polymorphisms on the survival of pediatric patients with acute lymphoblastic leukemia. Leuk Res Treatment. 2012;2012:292043.
13. Antonio-Vejar A, Del Moral-Hernández O1, Alarcón-Romero LC1, et. al. Ethnic variation of the C677T and A1298C polymorphisms in the methylenetetrahydrofolate-reductase (*MTHFR*) gene in southwestern Mexico. Genet Mol Res. 2014;13:7950-7.
14. Ramos MA, Mares RE, Avalos ED, et. al. Pharmacogenetic screening of N-acetyltransferase 2, thiopurine s-methyltransferase, and 5,10-methylene-tetrahydrofolate reductase polymorphisms in Northwestern Mexicans. Genet Test Mol Biomarkers. 2011; 15:351-5.
15. Guéant-Rodriguez RM, Guéant JL, Debard R, et.al. Prevalence of methylenetetrahydrofolate reductase 677T and 1298C alleles and folate status: a comparative study in Mexican, West African, and European populations. Am J Clin Nutr. 2006;83:701-7.
16. Robien K, Ulrich CM. 5,10-Methylenetetrahydrofolate reductase polymorphisms and leukemia risk: a HuGE mini-review. Am J Epidemiol. 2003;157:571-82.
17. Taşbaş O, Borman P, Gürhan Karabulut H, Tükün A, Yorgancıoğlu R. The frequency of A1298C and C677T polymorphisms of the methylenetetrahydrofolate gene in Turkish patients with rheumatoid arthritis: Relationship with methotrexate toxicity. Open Rheumatol J. 2011;5:30-5.
18. NCCN Guidelines Version 2.0 2015. Acute Lymphoblastic Leukemia. Available at: http://www.nccn.org/professionals/physician_gls/pdf/all.pdf (Accessed January 5, 2016).
19. Kantarjian HM, O'Brien S, Smith TL, et al. Results of treatment with Hyper-CVAD, a dose-intensive regimen, in adult acute lymphocytic leukemia. J Clin Oncol. 2000;18:547-61.
20. Samara SA, Irshaid Y, Mustafa KN. Association of MDR1 C3435T and RFC1 G80A polymorphisms with methotrexate toxicity and response in Jordanian rheumatoid arthritis patients. Int J Clin Pharmacol Ther. 2014;52:746-55.
21. Huang X, Gao Y, He J, et al. The association between RFC1 G80A polymorphism and cancer susceptibility: Evidence from 33 studies. J Cancer. 2016;7:144-52.
22. Leyva-Vázquez MA, Organista-Nava J, Gómez-Gómez Y, Contreras-Quiroz A, Flores-Alfaro E, Illades-Aguiar B. Polymorphism G80A in the reduced folate carrier gene and its relationship to survival and risk of relapse in acute lymphoblastic leukemia. J Investig Med. 2012;60:1064-7.
23. Kumagai K, Hiyama K, Oyama T, Maeda H, Kohno N. Polymorphisms in the thymidylate synthase and methylene tetrahydrofolate reductase genes and sensitivity to the low-dose methotrexate therapy in patients with rheumatoid arthritis. Int J Mol Med. 2003;11:593-600.
24. Mandola MV, Stoehlmacher J, Zhang W, et al. A 6bp polymorphism in the thymidylate synthase gene causes message instability and is associated with decreased intratumoral TS mRNA levels. Pharmacogenetics. 2004;14:319-427.
25. Yates Z, Lucock M. G80A reduced folate carrier SNP modulates cellular uptake of folate and affords protection against thrombosis via a non homocysteine related mechanism. Life Sci. 2005; 77:2735-42.
26. Dervieux T, Kremer J, Lein DO, et al. Contribution of common polymorphisms in reduced folate carrier and gamma-glutamyl hydrolase to methotrexate polyglutamate levels in patients with rheumatoid arthritis. Pharmacogenetics. 2004;14:733-9.
27. Dervieux T, Furst D, Lein DO, et al. Polyglutamation of methotrexate with common polymorphisms in reduced folate carrier, aminoimidazole carboxamide ribonucleotide transformylase, and thymidylate synthase are associated with methotrexate effects in rheumatoid arthritis. Arthritis Rheum. 2004;50:2766-74.

28. Wessels JA, de Vries-Bouwstra JK, Heijmans BT, et al. Efficacy and toxicity of methotrexate in early rheumatoid arthritis are associated with single-nucleotide polymorphisms in genes coding for folate pathway enzymes. Arthritis Rheum. 2006;54: 1087-95.
29. Ranganathan P, Culverhouse R, Marsh S, et al. Single nucleotide polymorphism profiling across the methotrexate pathway in normal subjects and patients with rheumatoid arthritis. Pharmacogenomics. 2004;5:559-69.
30. Whetstine JR, Gifford AJ, Witt T, et al. Single nucleotide polymorphisms in the human reduced folate carrier: characterization of a high-frequency G/A variant at position 80 and transport properties of the His(27) and Arg(27) carriers. Clin Cancer Res. 2001;7:3416-22.
31. Dervieux T, Furst D, Lein DO, et al. Pharmacogenetic and metabolite measurements are associated with clinical status in patients with rheumatoid arthritis treated with methotrexate: results of a multicenter crossectional observational study. Ann Rheum Dis. 2005;64:1180-5.
32. Dervieux T, Greenstein N, Kremer J. Pharmacogenomic and metabolic biomarkers in the folate pathway and their association with methotrexate effects during dosage escalation in rheumatoid arthritis. Arthritis Rheum. 2006;54:3095-103.
33. de Deus DM, de Lima EL, Seabra Silva RM, Leite EP, Cartaxo Muniz MT. Influence of methylenetetrahydrofolate reductase C677T, A1298C, and G80A polymorphisms on the survival of pediatric patients with acute lymphoblastic leukemia. Leuk Res Treatment. 2012;2012:292043.
34. Ruiz-Argüelles GJ, Coconi-Linares LN, Garcés-Eisele J, Reyes-Nuñez V. Methotrexate-induced mucositis in acute leukemia patients is not associated with the MTHFR 677T allele in Mexico. Hematology. 2007;12:387-91.
35. Cui G, Ding H, Xu Y, Li B, Wang DW. Applications of the method of high resolution melting analysis for diagnosis of Leber's disease and the three primary mutation spectrum of LHON in the Han Chinese population. Gene. 2013;512:108-12.
36. Frueh FW, Noyer-Weidner M. The use of denaturing high-performance liquid chromatography (DHPLC) for the analysis of genetic variations: impact for diagnostics and pharmacogenetics. Clin Chem Lab Med. 2003;41:452-61.
37. Taja-Chayeb L, Vidal-Millán S, Gutiérrez O, Ostrosky-Wegman P, Dueñas-González A, Candelaria M. Importance of the polymorphisms of the Thiopurine S-methyltransferase Gene (TMPT) determination in Mexican Mestizo patients with acute lymphoblastic leukemia (ALL). Med Oncol. 2008;25:56-62.
38. Xiao W, Oefner PJ. Denaturing high-performance liquid chromatography: A review. Hum Mutat. 2001;17:439-74.
39. O'Donovan MC, Oefner PJ, Roberts SC, et al. Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection. Genomics. 1998;52:44-9.
40. Steinmetz LM, Davis RW. High-density arrays and insights into genome function. Biotech Genet Eng Rev. 2000;17:109-47.