UNIVERSIDAD AUTÓNOMA DE GUERRERO

UNIDAD ACADÉMICA DE CIENCIAS QUÍMICO BIOLÓGICAS
UNIDAD ACADÉMICA DE MEDICINA
MAESTRÍA EN CIENCIAS BIOMÉDICAS

"POLIMORFISMOS EN EL GEN DE LA DIHIDROFOLATO REDUCTASA Y RESPUESTA AL METOTREXATO EN LEUCEMIA LINFOBLÁSTICA AGUDA EN MÉXICO"

T E S I S

Q U E P A R A O B T E N E R E L G R A D O D E

MAESTRÍA EN CIENCIAS BIOMÉDICAS

P R E S E N T A :

YAZMÍN GÓMEZ GÓMEZ

DIRECTOR DE TESIS:

DR. MARCO ANTONIO LEYVA VÁZQUEZ

CHILPANCINGO GUERRERO, AGOSTO 2009.

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APROBACIÓN DE TESIS

En la ciudad de Chilpancingo, Guerrero, siendo los 10 días del mes de julio de dos mil nueve, se reunieron los miembros del Comité Tutoral designado por la Academia de Posgrado de la Maestría en Ciencias Biomédicas, para examinar la tesis titulada **"Polimorfismos en el gen de la dihidrofolato reductasa y respuesta al metotrexato en leucemia linfoblástica aguda en México", presentada por** la alumna Yazmín Gómez Gómez, para obtener el Grado de **Maestría en Ciencias Biomédicas**. Después del análisis correspondiente, los miembros del comité manifiestan su aprobación de la tesis, autorizan la impresión final de la misma y aceptan que, cuando se satisfagan los requisitos señalados en el Reglamento General de Estudios de Posgrado e Investigación Vigente, se proceda a la presentación del examen de grado.

El Comité Tutoral

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“Polymorphisms in the dihydrofolate reductase gene and response to methotrexate in acute lymphoblastic leukemia in Mexico”

Este trabajo se realizó en el Laboratorio de Biomédicina Molecular de la Unidad Académica de Ciencias Químico Biológicas de la Universidad Autónoma de Guerrero, en la Ciudad de Chilpancingo Gro, México.

Bajo la dirección de

Dr. Marco Antonio Leyva Vázquez

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Con la colaboración de

Instituto Estatal de Cancerología “Arturo Beltrán Ortega”
Dr. Marco Antonio Terán Porcayo
Dra. Ana Bertha Rivera Ramírez

Durante el período en que cursó la Maestría en Ciencias Biomédicas, la C. Yazmín Gómez Gómez, recibió beca del CONACYT.

El presente trabajo fue enviado para su publicación en la revista Leukemia and Lymphoma, por lo que el formato en el que se presenta es de acuerdo a los lineamientos de la revista.

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ÍNDEX

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Polymorphisms in the dihydrofolate reductase gene and response to methotrexate in acute lymphoblastic leukemia in Mexico

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Running title: Dihydrofolate reductase leukemia Mexico

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ABSTRACT

The presence of polymorphisms in regulatory regions causes increased expression of dihydrofolate reductase (DHFR), and it is associated with methotrexate (MTX) treatment adverse events. A case control study was conducted on 70 children with acute lymphoblastic leukemia (ALL) and 140 children without disease. We used the polymerase chain reaction restriction fragment length polymorphism method to genotype the –A317G and C829T polymorphisms in the DHFR and assess the association with lack of response to methotrexate treatment. The polymorphisms and lack of response to MTX showed an association ($p<0.05$). The genotype GG [odds ratio (OR), 8.55; confidence interval (CI) 95%, 1.84-39.70] and TT [odds ratio (OR), 14.0; confidence interval (CI) 95%, 1.13-172.63] were associated with lack of response to methotrexate. Our data suggests that children with ALL carriers of the G and T allele have more risk to have a lack of response to methotrexate treatment.

Keywords: Acute lymphoblastic leukemia, dihydrofolate reductase, lack of response to methotrexate, single nucleotide polymorphism, –A317G polymorphism, C829T polymorphism.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a hematologic neoplasia characterized by clonal proliferation of immature lymphoid cells, the result of the malignant transformation of a lymphoid progenitor cell [1-3]. In Mexico, acute leukemia is considering a problem of public health, it represents the fourth position of mortality of all neoplasic malignancies in children younger than fifteen years [4-8]. In 2005, leukemia was the second cause of mortality in state of Guerrero in children younger than fifteen years, according to Institute National of Statistics Geography and Computing [9].

For five decades, methotrexate (MTX) was introduced to the oncology clinical as treatment for patients with ALL and is presently used in the treatment of other neoplasic diseases, including osteosarcoma, breast cancer, head and neck cancers, and non-Hodgkin's lymphoma [10-13]. MTX is a folic acid antagonist, its efficacy as an antineoplasic treatment is largely attributed to the high affinity of MTX for dihydrofolate reductase (DHFR) [14-16]. DHFR (EC 1.5.1.3) catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF). The major mechanism of MTX action involves competitive inhibition of DHFR, this leads to the impaired regeneration of THF from DHF; essential for the biosynthesis of purines and thymidylate, thus it also blocks the *novo* synthesis of DNA and cell growth [17-19].

A subset of patients can develop adverse events due to the drug or lack response to MTX, which may hamper the efficacy of treatment. The MTX can cure approximately 80% of the children with B-lineage ALL and fail in 20%, an event frequently related with T-lineage ALL [12, 13, 20, 21]. The multiple mechanisms that can lead to clinical failure to MTX are; DHFR overexpression, impaired intracellular transport of MTX and decreased levels of reduced folate carrier (RFC) at the cell membrane [22-25].

Changes in the level of DHFR expression and consequently in the sensitivity to MTX can also be due to polymorphisms (SNPs), particularly those located in the regulatory elements. The SNP C829T is located at the 223 nucleotide downstream from the stop codon between the first and second polyadenylation sites in the 3´UTR of the DHFR gene which leads to the stability of the mRNA [26, 27]. A recent study reported the SNP –A317G in the DHFR promoter region which conferred them higher transcriptional activity [28].

Mexico does not have studies of the frequency of the –A317G and C829T SNPs in the DHFR gene, or the association with lack of response to methotrexate treatment in ALL

children. In the present study our objective was to assess the association of the SNPs in the DHFR and lack response to MTX in children with ALL, and also to determine the genotypic and allelic frequencies on children with and without ALL. Our results suggest that the identification of the SNPs –A317G and C829T in the DHFR gene, target of MTX, may provide information about the response to methotrexate treatment in ALL children.

MATERIALS AND METHODS

Study population

A case control study was conducted out in the Cancer Institute of the State of Guerrero, a regional concentration Hospital located in Acapulco, Guerrero, Mexico. The cases were 70 children diagnosed with ALL through bone marrow aspirate based on French-American-British morphological criteria and cytochemical assays, between Augusts 2005 and December 2008 in the pediatric oncology. The patients underwent treatment with MTX according to Cancer Institute protocols 96091, 96092 or CIE-10:C9.1.0 [29], and were classified in MTX responders and non-responders, considering >25% of blast in bone marrow as lack response treatment, after receiving MTX treatment. The controls were 140 children without ALL (4.0-10.0 x 10^3 Leukocytes/mm^3) that referred not having a family history of leukemia. Both groups of study had of 1-18 years of age, both sexes and with residence in The State of Guerrero. Informed consent was obtained from all the individuals or their guardians, after a detailed briefing of the study purposes. This study was approved by the ethical committee of the Cancer Institute of the State of Guerrero.

Specimen collection

A blood sample was taken from the 210 participants, and placed in tubes with anticoagulant. Leukocytes were purified from the whole blood sample by a selective osmotic lysis of erythrocytes and the leukocyte genomic DNA was extracted by the phenol-chloroform technique [30, 31].

Genotyping of the -A317G and C829T polymorphism in the DHFR

The –A317G polymorphisms (dbSNP; rs408626) was detected using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The amplification of a 400 bp fragment by PCR was according to that described by Dulucq *et al*., 2008, with some modifications. Genomic DNA (100ng) was amplified in 1X PCR Buffer, 0.2 mM of dNTPs mixture (Applied Biosystems, Foster City, CA, U.S.A.), 0.4 µM each of forward primer (5'-GTAGGTTC TGTCTGGGACTGG-3') and reverse primer (5'-GCAGCTTTCTTCTAGTCACCC-3'), 1 unit of Taq DNA polymerase recombinant (Invitrogen™ life technologies, U.S.A.) and 1.5 mM $MgCl_2$ in a final volume of 25 µL. The PCR conditions were an initial denaturation of 2 min at 94°C, 40 cycles of 30 s at 94° C, 45 s at 65° C, 45 s at 72° C and a final extension of 5 min at 72 ° C. The PCR products were

digested with 4 units of the *Hinf* I enzyme (Invitrogen^{TM} life technologies, U.S.A.) in a final volume of 15 μL. Individuals with the AA genotype presented two fragments (266 bp and 134 bp), individuals with the AG genotype presented four fragments (266 bp, 134 bp, 83 bp, 51 bp) and those with the GG genotype three fragments (266 bp, 83 bp, 51 bp). The digested products were observed in 10% acrylamide gel.

The C829T polymorphism (Goto et al., 2001) in the 3´UTR region was detected by PCR-RFLP. The PCR primers (forward 5'-TCCAAGACCC CAACTGAGTC-3 ', reverse 5 '-TCACTGTTACAAACAAGGTGG C-3') were designed to amplify a fragment of 243 pb from the sequence accesses GenBank J00139, using a reaction mixture similar to that used for the -A317G polymorphism, differing only in the amount of enzyme a 0.5 units of Taq DNA polymerase recombinant (Invitrogen^{TM} life technologies, U.S.A.). Amplification was performed by an initial denaturation of 5 min at 95°C, 40 cycles of 15 s at 95° C, 45 s at 54° C, 30 s at 72° C and a final extension of 7 min at 72 ° C. The PCR products were digested with 3 units of the *TspR* I enzyme (New England Biolabs, Berverly, MA U.S.A.), in a final volume of 15 μL. Fragments of 202 bp and 41 bp identified the CC genotype, 243 bp, 202 bp and 41 bp the CT genotype and a 243 bp fragment the TT genotype. The digested products were observed in a 3% agarose gel.

Statistical analysis

The chi-square or Fisher's exact test was used for comparison of genotype and allele frequencies between groups and different populations. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by using logistic regression to estimate the risk of association of response to MTX treatment in ALL children with -A317G and C829T polymorphism genotypes and different covariables. The Hardy-Weinberg test was used to determine the genetic equilibrium in the group without ALL. A value of $p<0.05$ was considered statistically significant. All statistical analysis was performed using STATA software version 9.2.

RESULTS

General characteristics of the population and frequency of polymorphisms

The age range at diagnosis of 70 children with ALL was 1 to 18 years with an average of 7.65 ± 4.67 years; 64.29% were male and 35.71% female. The leukocyte count was of 1,000 leukocytes/mm^3 as minimum and a maximum of 290.000 leukocytes/mm^3 with a median of 13 000 leukocytes/mm^3. 57.14% of the children were treated for over a year, 92.86% received a dose of 0.5-3.0 g/m2 of MTX weekly. However, 68.57% of the children had relapses during treatment. Until 2008 there is a record of 40 deaths of the 70 children with ALL included in our study (57.14%). Of the children without ALL; 53.57% was represented by males and 46.43% by females. The average age was of 9.99±5.49 years (Table I).

In children with ALL, the genotypic distribution of the -A317G polymorphism was: AA (20.0%), AG (47.1%) and GG (32.9%). The frequency of A and G allele was 0.436 and 0.564, respectively. In children without ALL genotypic frequencies were in agreement as expected by the Hardy-Weinberg equilibrium (p= 0.184), being the most frequent genotype AG with 43.6% (Table II).

Table I. General characteristics of the population and clinical data of children with acute lymphoblastic leukemia

Variable	Children with ALL n= 70	Children without ALL n= 140
Age (years)	7.65 ± 4.67	9.99± 5.49
Number of leukocytes/mm^3	13000 (5400-39000)[a]	8000 (7000-9000)[a]
Sex		
Male	45 (64.29)	75 (53.57)
Female	25 (35.71)	65 (46.43)
Status of participants		
Live	30 (42.86)	140 (100)
Dead	40 (57.14)	0
Weekly MTX dose		
< 0.5 g/m^2	3 (4.29)	0
0.5- 3.0 g/m^2	65 (92.86)	0
> 3.0 g/m^2	2 (2.86)	0
Time on MTX		
< one years	30 (42.86)	0
> one years	40 (57.14)	0
Relapse during treatment with MTX		
No	22 (31.43)	0
Yes	48 (68.57)	0

Data indicate n (%); mean ± standard deviation (SD), [a] median (Percentiles 25-75)

The genotype and allele frequencies were statistically different between study groups p <0.001, p = 0.013, respectively. The genotypic distribution of the C829T polymorphism in children with ALL was: CC (14.3%), CT (75.7%) and TT (10.0%). The frequency of C and T allele was 0.521 and 0.479, respectively. Children without ALL had higher frequency of CT genotype (70.0%). However, genotypic frequencies were not found in genetic equilibrium according to the law of Hardy-Weinberg (p <0.001). It was observed that the high heterozygosity for the C829T polymorphism was a reason why the gene balance was lost, so that 20% of the samples were submitted for sequencing, yielding reproducible results of heterozygosis. There were no differences in genotype and allele frequencies between children with and without ALL p= 0.380 and p= 0.350, respectively (Table II).

Table II. Genotype distribution and allele frequency of -A317G and C829T polymorphisms of DHFR in children with and without ALL

Population (n)	Genotype distribution			*p*-value	Allele frequency		*p*-value	*p*-value *HWE*
	A/A	A/G	G/G		A	G		
-A317G polymorphisms								
ALL children (70)	14 (20.00%)	33 (47.14%)	23 (32.86%)		61 (43.57%)	79 (56.43%)		
Children without ALL (140)	49 (35.00%)	61 (43.57%)	30 (21.43%)	<0.001[a]	159 (56.79%)	121 (43.21%)	0.013[a]	0.184
	C/C	C/T	T/T		C	T		
C829T polymorphisms								
ALL children (70)	10 (14.29%)	53 (75.71%)	7 (10.00%)		73 (52.14%)	67(47.86%)		
Children without ALL (140)	31 (22.14%)	98 (70.00%)	11 (7.86%)	0.380[a]	160 (57.14%)	120 (42.86%)	0.350[a]	<0.001

[a]*p* value was obtained by chi-square test. *HWE* (Hardy-Weinberg equilibrium)

Response to MTX in relation to genetic polymorphisms in children with ALL

In a logistic regression analysis, we found an association between polymorphisms, A317G, C829T and the lack of response to MTX (p <0.05). Those children with ALL carrying the GG genotype of the -A317G polymorphism had 8.6 times the risk of not responding to MTX compared to carriers of AA genotype (p = 0.006). Those carrying the C829T polymorphism TT genotype had 14 times the risk of not responding to treatment with MTX compared to carriers of the CC genotype (p = 0.039) (Table III).

Table III. Association of -A317G and C829T polymorphisms in DHFR gene with the lack of response the MTX

	n	%	OR	CI 95%	*p-value*[*]
–A317G genotype					
A/A	14	20.00	1.00		
A/G	33	47.14	4.8	1.26-18.24	0.021
G/G	23	32.86	8.55	1.84-39.70	0.006
	70	100.0			
C829T genotype					
C/C	10	14.29	1.00		
C/T	53	75.71	6.5	1.47-28.67	0.013
T/T	7	10.00	14	1.13-172.63	0.039
	70	100.0			

Odds ratio (OR); 95% confidence interval (CI); [*]*p* obtained the logistic regression analysis, taking the referent to AA and CC genotypes.

Making a distribution of genotype and allele frequency of polymorphisms in relation to the MTX responders and non-responders to, it was found that 10.4% of children with ALL that were MTX non-responders to were carriers of the AA genotype, 50.0% of AG genotype and 39.58% of the GG genotype. Compared to MTX responders that showed the AA genotype in 40.9%, genotype AG in 40.9% and the GG genotype in a 18.2% (Table IV).

Table IV. Genotype distribution and allele frequency of -A317G and C829T polymorphisms of DHFR children with ALL MTX responders and non-responders

Genotypes	Responder's n (%)	Non-responders n (%)	*p-value**
-A317G			
A/A	9 (40.91)	5 (10.42)	
A/G	9 (40.91)	24 (50.00)	
G/G	4 (18.18)	19 (39.58)	0.014[a]
	22 (100.0)	48 (100.0)	
Allele			
A	27 (61.36)	34 (35.42)	
G	17 (38.64)	62 (64.58)	0.004[a]
	44(100.0)	96(100.0)	
Genotypes			
C829T			
C/C	7 (31.82)	3 (6.25)	
C/T	14 (63.64)	39 (81.25)	
T/T	1 (4.54)	6 (12.50)	0.015[a]
	22 (100.0)	48 (100.0)	
Allele			
C	28 (63.64)	45 (46.88)	
T	16 (36.36)	51 (53.12)	0.048[a]
	44 (100.0)	96 (100.0)	

Odds ratio (OR); 95% confidence interval (CI); [*]*p* value was obtained by chi-square test; [a] Significant $p<0.05$

Analyzing the MTX non-responders group in relation to the C829T polymorphism it was observed that 81.25% were carriers of CT genotype, 12.50% had the TT genotype and only 6.25% carried the CC genotype. In contrast to responders in which 31.8% were carriers of the CC genotype, 63.6% of the CT genotype and 4.54% carriers of the TT genotype. Statistical differences in genotypic and allelic frequencies of both polymorphisms between MTX responders and non-responders were found ($p < 0.05$) (Table IV).

Other variables such as age, sex, leukocyte count and MTX doses received were not associated with response to MTX $p > 0.05$ (Table V).

Table V. Association the lack of response the MTX and the different covariants

	n	%	OR	IC 95%	*p-value*
Sex					
Female	25	35.71	1.00		
Male	45	64.29	1.38	0.49-3.92	0.540[a]
Age (years)					
1-6	34	48.57	0.54	0 .19-1.50	0.236
7-12	24	34.29	1.60	0.53-4.83	0.405
13-18	12	17.14	1.46	0.35- 6.03	0.600
Leukocytes at diagnosis					
1000-10000	29	41.43	1.36	0.48-3.85	0.561
10001-100000	34	48.57	0.54	0.19-1.50	0.236
100001-290000	7	10.00	3.00	0.34-26.56	0.323
Weekly MTX dose					
< 0.5 g/m^2	3	4.29	0.91	0.08-10.64	0.942
0.5-3.0 g/m^2	65	92.86	0.52	0.06-4.98	0.574
> 3.0 g/m^2	2	2.86	0	0	0

Odds ratio (OR); 95% confidence interval (CI), [a] taking the reference to female sex

DISCUSSION

Clinical and *in vitro* studies have demonstrated the involvement of polymorphisms in non-coding regions of the DHFR that increase the expression and induce lack of response to MTX [32]. This paper reports that the -A317G polymorphism in the promoter site and the C829T polymorphism in the 3'UTR of DHFR is associated with lack of response to methotrexate in ALL children.

The C829T polymorphism is not in Hardy-Weinberg equilibrium, 20% of these samples were subjected to genotyping sequencing, obtaining results similar to those reported by RFLP. This concludes that the study population is not in equilibrium for the C829T polymorphism, the used samples for this variant were the same as those used in the -A317G variant, is in Hardy-Weinberg genetic equilibrium. However, population studies are needed to determine the introduction of the mutation in our study population. The genotypic and allelic frequencies of the -A317G polymorphism were statistically different between children with and without ALL ($p < 0.001$, $P = 0.013$), suggesting that it may be associated with ALL.

In this study, children with ALL showed predominantly the heterozygous AG genotype (47.1%), a result similar to that reported by Dulucq *et al*., in a Canadian population where the AG genotype was reported as the most frequent in individuals with ALL (48.4%) [28]. However, the genotypic frequencies; AA (31.4%), AG (48.4%), GG (20.2%) and allelic frequencies; A (55.6%) and G (44.4%) reported by Dulucq are statistically different from those found in this study ($p = 0.040$, 0.014), as the Mexican population has an increased frequency of risky genotypes.

The genotypic and allelic frequencies of the C829T polymorphism reported in this study differ from those reported by Goto *et al.*, in the Japanese population with ALL, where the most frequently reported is the CC genotype (83.8%), followed by the CT genotype (10.8%) and the TT genotype (5.4%), and allelic frequencies of C (89.2%) and T (10.8%) ($p<0.001$) [27]. These data suggest that the Mexican population with ALL has an increased frequency of C829T and A317G polymorphisms. It is important to note that the geographical distribution, environmental exposure, polymorphism and short tandem repeat (STR), may be determinants of genetic variation from one population to another. According to reports by the National Institute of Genomic Medicine (INMEGEN), 65% of the genetic component of the Mexican population is unique and 22% of genes are shared with the African race [33]. The

differences in the genotypes between the Mexican population and the Japanese and Canadian population reflect our mestizo component of a mixture of Amerindian, Spanish and Africans; although with the Canadian population shared a similarity in the AG genotype [34-36].

Due to the little information on the frequency of the C829T and A317G polymorphisms in individuals with ALL, the frequencies obtained were compared with those reported by the authors mentioned above. The C829T polymorphism is the variant that has been studied in other disorders that involve the metabolic pathway of folate. However, it was not identified in non- Japanese American, Caucasians or Israeli population [37-39]. Suggesting that the C829T polymorphism is found more frequently in patients with ALL than in those with other disorders in which the DHFR is involved.

Dulucq *et al*., 2008, presents evidence that the -A317G polymorphism in the promoter of the DHFR confers high transcriptional activity associated with lack of response to MTX in patients with ALL. Matheson *et al*., 2007 showed that ALL cell lines resistant to MTX have disruptions in the transcriptional level of DHFR [40]. These reports support the theory that the molecular mechanism that may be happening in our group of individuals with ALL that are MTX non-responders, are carriers of allele G (89.58%).

There is evidence supporting that the C829T polymorphism is located adjacent to a binding site of a miRNA in the 3'UTR region of the DHFR affecting their function and resulting in the overexpression of the DHFR by mRNA stability [41]. This is further evidence that supports that the C829T polymorphism is involved in conferring lack of response to MTX by overexpression of the DHFR in patients with ALL carrying the T allele. Goto *et al*., 2001, reports the C829T polymorphism in the 3'UTR of DHFR in patients with ALL is associated with overexpression of the enzyme mainly in those carrying the TT genotype. In the present study, 93.75% of non-responders to MTX were carriers of allele T in conjunction with previous experimental studies which identifies the T allele as of risk.

The literature suggests that with current treatments, 80% of children with ALL are cured [32]. However, in the population with ALL in our study, 57.14% have died even when they were treated with MTX. This indicates that the presence of polymorphisms, A317G, C829T and its strong association with the lack of response to MTX ($p < 0.05$) may be a factor that led to more than 50% of deaths in patients with ALL included in this study.

The response to chemotherapeutic treatment is influenced by many other factors, in this study the applied dose of MTX and leukocyte count did not have significant influence on the response to MTX ($p > 0.05$), coinciding with those reported by Dervieux et al., and Dulucq et al. The age and sex did not influence the response to MTX, a fact also reported by Kim et al., that confirming that these variables did not influence the response to chemotherapy [28, 42, 43].

In conclusion, the -A317G and C829T polymorphisms are strongly associated with a lack of response to MTX presenting a higher risk of failure to MTX treatment in those patients with ALL carrying the GG and TT genotypes compared with those carrying the AA and CC genotypes, respectively. However, it would it is too early to say that in the Mexican population with ALL the sole or main cause of lack of response to MTX is to carry the -A317G and C829T polymorphisms, since it is known that intracellular levels of DHFR are regulated by a large number of factors. So cytotoxicity testing would have to be done, identification of other polymorphisms in other enzymes involved in the metabolism of MTX, identification of the loss of function of miRNA by polymorphisms in the regulatory 3'UTR regions, measuring levels of mRNA and the DHFR protein to check that their overexpression is indeed associated with lack of response to MTX in Mexican patients with ALL.

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