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Involvement of *MTHFR* and *TPMT* genes in susceptibility to childhood acute lymphoblastic leukemia (ALL) in Mexicans

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Abstract

Background: Folate metabolism plays an essential role in the processes of DNA synthesis and methylation. Deviations in the folate flux resulting from single-nucleotide polymorphisms in genes encoding folate-dependent enzymes may affect the susceptibility to leukemia. This case-control study aimed to assess associations among *MTHFR* (C677T, A1298C) and *TPMT* (*2, *3A) mutations as well as to evaluate the synergistic effects of combined genotypes for both genes. Therefore, these genetic variants may lead to childhood acute lymphoblastic leukemia (ALL) susceptibility, in a Mexican population study.

Methods: DNA samples obtained from 70 children with ALL and 152 age-matched controls (range, 1–15 years) were analyzed by real-time reverse transcription polymerase chain reaction (RT-qPCR) to detect *MTHFR* C677T and A1298C and *TPMT**2 and *TPMT**3A genotypes.

Results: The frequency of the *MTHFR* A1298C CC genotype was statistically significant (odds ratio [OR], 6.48; 95% 95% confidence intervals [CI], 1.26–33.2; p=0.025). In addition, the combined 677CC+1298AC genotype exhibited a statistically significant result (OR, 0.23; 95% CI, 0.06–0.82; p=0.023). No significant results were obtained from the *MTHFR* (C677T CT, C677T TT) or *TPMT* (*2, *3A) genotypes. More importantly, no association between the synergistic effects of either gene (*MTHFR* and/or *TPMT*) and susceptibility to ALL was found.

Conclusions: The *MTHFR* A1298C CC genotype was associated with an increased risk of developing childhood ALL. However, a decreased risk to ALL with the combination of *MTHFR* 677CC+1298AC genotypes was found.

Keywords: acute lymphoblastic leukemia; *MTHFR*; *TPMT*.

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Introduction

The precise mechanism of tumorigenesis is not yet resolved, but it is likely to include genetic and environmental interactions as main risk factors [1]. Kandy and Vadakedath [2] describe that folic acid and methionine play a significant role in DNA methylation and nucleotide synthesis. Therefore, folate pathway might participate in the development of carcinogenic processes. One of the main components of folate pathway is encoded by the methylene-tetrahydofolate-reductase (*MTHFR*) gene, which is located on chromosome 1p36.3 and consists of 11 exons. The gene product allows the generation of methyl donors in the synthesis of *S*-adenosyl-methionine (SAM), which is involved in DNA methylation reactions [3].

Skibola et al. [4] identified more than 20 mutations in *MTHFR*, including mutations that caused the transitions C677T (Ala22Val) and A1298C (Glu429Ala), which have been well characterized due to the severe enzymatic deficiency they cause.

An association between functional polymorphisms in the *MTHFR* gene and leukemogenesis has been demonstrated [5]; also, a folate deficiency that increased DNA damage and tumorigenicity *in vitro* has been proposed [6].

However, findings regarding *MTHFR* polymorphisms and risk to lymphoma, cervical cancer, and acute lymphoblastic leukemia (ALL) have been reported to differ among populations [4, 7–9].

Moreover, Arenas et al. [10] suggested for the first time, that SAM (a thiopurine methyltransferase [*TPMT*] cofactor) and an *MTHFR* enzyme involved in *MTHFR* biosynthesis influence the *TPMT* activity [10]. Furthermore, it has been proposed that the binding of SAM stabilizes the enzyme's three-dimensional structure and thus affects the therapy's outcome [11]. This is due to *TPMT*, which regulates the elimination of anticancer drugs including azathioprine, 6-mercaptopurine, and 6-thioguanine [12].

The TPMT enzyme is encoded by the *TPMT* gene, which is located on chromosome 6p22.3 and consists of 10 exons. Single-nucleotide polymorphisms (SNPs) in the *TPMT* gene, including *TPMT*2* (G238C), *TPMT*3A* (G460A and A719G), *TPMT*3B* (G460A), and *TPMT*3C* (A719G) have been described [13]. The mutations G460A and A719G are usually inherited together as the *TPMT*3A* allele; thus, heterozygote or homozygote individuals for these variants might be exceptionally sensitive to the myelosupressive drug effects and the carcinogenic potential risks associated [13].

To date, no association among the *TPMT*2* and *TPMT*3C* polymorphisms and ALL susceptibility has been reported in any previous research study [14].

Therefore, to the best of our knowledge, this present study is the first report to evaluate the combined effects of *MTHFR* and *TPMT* SNPs and susceptibility to ALL in Mexicans. Consequently, this case-control study aimed to assess associations of the *MTHFR* (C677T, A1298C) and *TPMT* (*2, *3A) polymorphisms and to evaluate the synergistic effects of combined genotypes for both genes with susceptibility to childhood ALL in a Mexican population study.

Materials and methods

Patients

Pediatric patients were recruited from two hospitals in México (El Centro de Oncología Pediátrica de Baja California and El Centro Estatal de Cancerología de Durango) – these two centers are located in cities from the northern region of the country. The case group included 70 children with medical diagnosis of ALL (41 boys and 29 girls) with a mean age of 6.9 years (range, 1–15 years).

The age-matched control group included 152 randomly selected individuals (88 boys and 64 girls) with a mean age of 6.7 years. Subjects with history of malignant neoplasms were excluded. Both groups were recruited from March 2013 to July 2014. Children's parents or legal tutors were previously informed about the study's aims, and they provided written consent to participate.

This study was reviewed and approved by the Ethics and Research Committees from both hospitals, in accordance to the ethical principles of the Declaration of Helsinki [15].

MTHFR and *TPMT* genotyping

From each volunteer, 5 mL of peripheral venous blood was collected in EDTA-supplemented tubes. Genomic DNA was extracted from whole venous blood using a QIAmp DNA Blood Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA integrity was confirmed by 1% agarose gel electrophoresis and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Genotyping was carried out using semiquantitative real-time polymerase chain reaction (qRT-PCR) in a StepOne Real-Time PCR system (v.2.2; Applied Biosystems, Foster City, CA, USA) under standard conditions. MGB TaqMan probes were used to identify *MTHFR* C677T (C_1202883_20), *MTHFR* A1298C (C_850486_20), *TPMT*2* (C_12091552_30), and *TPMT*3A* (C__19567_20, C_30634116_20) polymorphisms.

Statistical analysis

Analysis of the Hardy-Weinberg equilibrium (HWE) for genotype distribution was calculated using an exact test, the χ^2 test. Pearson χ^2 test was also used to estimate genotypic differences and allele frequencies between case and control groups. The odds ratios (ORs) of *MTHFR* and *TPMT* genotypes were used to test for statistically significant associations between these SNPs and ALL susceptibility. All analyses were carried out using the Statistical Analysis System software v.8.2 (SAS Institute, Cary, NC, USA). The threshold for statistically significant differences was set at $p<0.05$.

The sample size was determined based on the frequency of exposure among cases and controls with 95% confidence intervals (CI) and $\beta=0.80$ along with two controls for each case; these estimates were based on the formula by Pértega Diaz and Fernández [16].

Results

The genotype distributions of the *MTHFR (*C677T and A1298C) and *TPMT* (*2 and *3A) polymorphic loci did not deviate from HWE, except for *MTHFR* 1298 genotype in the ALL group. The genotypic frequencies for the *MTHFR* A1298C polymorphism are shown in Table 1; the frequency of the CC genotype for the A1298C polymorphism was statistically significant (OR=6.48; 95% CI, 1.26–33.2; p=0.025), suggesting an association of this mutation with ALL.

In the case of *MTHFR* C677T (also shown in Table 1), none of the genotypes showed statistically significant differences between the case and the control groups.

Table 1: Frequencies of *MTHFR* C677T and A1298C polymorphisms in the study subjects.

Genotype	ALL patients, n (%)	Controls, n (%)	OR (95% CI)	p-Value
MTHFR C677T				
CC	22 (31.4)	42 (27.6)	1.00[a]	
CT	36 (51.4)	72 (47.4)	0.95 (0.50–1.83)	0.889
TT	12 (17.2)	38 (25.0)	0.60 (0.26–1.38)	0.231
CT+TT	48 (68.6)	110 (72.4)	0.83 (0.45–1.54)	0.561
C allele	0.57	0.51		
T allele	0.42	0.49		
MTHFR A1298C				
AA	50 (71.4)	108 (71.0)	1.00[a]	
AC	14 (20.0)	42 (27.7)	0.72 (0.36–1.44)	0.351
CC	6 (8.60)	2 (1.30)	6.48 (1.26–33.2)	0.025
AC+CC	20 (28.6)	44 (29.0)	0.98 (0.53–1.84)	0.954
A allele	0.81	0.84		
C Allele	0.19	0.16		

[a]As a reference group. Allelic frequencies=(homozygote alleles×2+heterozygote alleles)/total subjects×2.

Therefore, this variant is not associated with either risk or protective effect on ALL in Mexicans.

Further analysis of the combined effect for the *MTHFR* C677T and *MTHFR* A1298C genotypes was performed (Table 2). This analysis revealed that the combined 677CC+1298AC genotypes resulted in the haplotype with protective effect on ALL (OR=0.23; 95% CI, 0.06–0.82; p=0.023, corresponding to a reduced risk of 4.3-fold with 95% CI, 1.2–16.7).

Surprisingly, our data revealed no association of the *TPMT**2 and *TPMT**3A polymorphisms with childhood ALL in this Mexican sample (Table 3).

To investigate the synergistic effect for both genes in association with leukemia, two analyses were implemented: first, the *MTHFR* C677T with the *TPMT**2 and *TPMT**3A polymorphisms; second, the *MTHFR* A1298C with the *TPMT* genotypes. Both analysis combinations showed no significant associations to ALL.

Discussion

Herein, we detected a significant association between the *MTHFR* 1298CC genotype and the risk to ALL (OR=6.48; 95% CI, 1.26–33.2; p=0.025). Also, the Hardy-Weinberg test showed a marked equilibrium in the sample of controls; however, a deviation from the HWE arose from the polymorphism *MTHFR* A1298C in the case group, suggesting a real association between its CC genotype and ALL disease [20, 21].

Moreover, this study found a protective effect of the combined genotypes of *MTHFR* 677CC+1298AC. Such findings suggest that children with the wild-type genotype for the variant 677 in combination with the heterozygous genotype for 1298 of *MTHFR* polymorphisms appear to have a decreased susceptibility to ALL (OR=0.23; 95% CI, 0.06–0.82; p=0.023), whereas the *MTHFR* 677CT, *MTHFR* 677TT, and *TPMT**2 and *TPMT**3A genotypes did not significantly influence the risk in this Mexican population. In addition, the synergistic effect for both genes (*MTHFR* and *TPMT*) showed no association to ALL susceptibility. Thus, this present study is the first report to evaluate the combined effects of *MTHFR* and *TPMT* variants and susceptibility to ALL in Mexican patients.

Concerning gene association to ALL, our results are in concordance with those of Li et al. [22], who concluded that the A1298C polymorphism (AC heterozygous genotype) represents a high risk factor to ALL in Chinese population (OR, 2.08; 95% CI, 1.13–3.84). In addition, our findings are consistent with those reported by Zanrosso et al. [23], who studied Brazilian non-White children and concluded that

Table 2: Frequencies of combined *MTHFR* C677T and A1298C genotype polymorphisms in the study subjects.

MTHFR C677T/A1298C	ALL patients, n (%)	Controls, n (%)	OR (95% CI)	p-Value
	70 (100.0)	152 (100.0)		
677 CC/1298 AA	13 (18.6)	17 (11.2)	1.00[a]	
677 CC/1298 AC	4 (5.71)	23 (15.1)	0.23 (0.06–0.82)	0.023
677 CC/1298 CC	5 (7.14)	2 (1.30)	3.27 (0.54–19.6)	0.195
677 CT/1298 AA	25 (35.7)	54 (35.6)	0.61 (0.26–1.44)	0.254
677 CT/1298 AC	10 (14.3)	18 (11.8)	0.73 (0.25–2.09)	0.553
677 CT/1298 CC	1 (1.42)	0 (0.0)[b]	3.88 (0.14–103.1)	0.416
677 TT/1298 AA	12 (17.1)	37 (24.4)	0.42 (0.16–1.12)	0.083
677 TT/1298 AC	0 (0.0)[b]	1 (0.60)	0.43 (0.01–11.4)	0.615

[a]As s reference group. [b]Where zeros cause problems with computation of the odds ratio or its standard error, 0.5 was added to all cells (a, b, c, and d) [17–19].

Table 3: Frequencies of *TPMT***2* and *TPMT***3A* (460G>A and 719A>G) polymorphisms in the study subjects.

Genotype	ALL patients, n (%)	Controls, n (%)	OR (95% CI)	p-Value
TPMT				
*1/*1	69	149	1.00[a]	
*1/*2	1	3	0.72 (0.07–7.05)	0.777
*2/*2	0[b]	0[b]	2.15 (0.04–109.5)	0.702
*1	0.99	0.99		
*2	0.01	0.01		
*1/*1	65	138	1.00[a]	
*1/*3A	5	13	0.82 (0.28–2.39)	0.711
*3A/*3A	0[b]	1	0.70 (0.02–17.5)	0.831
*1	0.96	0.95		
*3A	0.04	0.05		

[a]As a reference group. Allelic frequencies=(homozygote alleles×2+heterozygote alleles)/total subjects×2. [b]Where zeros cause problems with computation of the odds ratio or its standard error, 0.5 was added to all cells (a, b, c, and d) [17–19].

the 1298C allele (CC homozygous genotype) is also a risk factor to ALL (OR, 2.01; 95% CI, 1.01–3.99).

Concerning this study, it is evident that in Mexican population, the frequency of this polymorphism is higher than those previously reported in other populations (OR, 6.48; 95% CI, 1.26–33.2).

Conversely, other studies failed to demonstrate this aforementioned association in different study populations [9, 24, 25]. Thus, we consider several reasons for these inconsistent results: first, the *MTHFR* genetic distribution in different populations; second, plasma folate levels associated with food intake and nutritional habits; third, the possibility that ALL susceptibility may be regulated by other folate-related genes such as *TPMT*, *RFC1*, *NNMT*, and *SHMT1* [14, 26, 27].

It is noticeable that the children with the CC homozygous genotype for the *MTHFR* 1298 polymorphism have risk of leukemia, but the children who present the *MTHFR* 677 CC (wild type) and the *MTHFR* 1298 CT (heterozygous) combination may have a protective effect on leukemia (OR=0.23; 95% CI, 0.06–0.82; p=0.023).

Taking into account the striking difference, it is possible that the homozygous individuals for *MTHFR* variants would have an insufficient intracellular distribution of folate, which is necessary to maintain the three-dimensional protein structure. As a result, the production of aberrant DNA methylation and nucleotide synthesis would increase the risk to ALL development [28, 29]. Therefore, low intracellular levels of folate products of *MTHFR* genetic variants represent an important topic for future research in this population, due to reports of scarce consumption of green leafy vegetables rich in folic acid in Mexicans [30].

Accordingly, folate metabolism is involved in carcinogenesis due to its participation in DNA methylation by 5-methyl-THF [31]. *MTHFR* is responsible for the reduction reaction from 5,10-methylenetetrahydrofolate (5,10-methylene-THF) to 5-methyltetrahydrofolate (5-methyl-THF) [31], the predominant circulatory form of folate [32]. Thus, Bagley and Selhub [33] examined the effect of *MTHFR* C677T genotype on red blood cell (RBC) folate content and its relative form of distribution from groups of 677TT (homozygous) and 677CC (wild type) individuals by HPLC method. They found that the folate content in RBCs of wild-type subjects (CC) is exclusively composed of 5-methyl polyglutamates, but in the case of homozygous subjects (TT), the folate content is composed of formylated THF polyglutamates. Therefore, this genotype is associated with an incorrect distribution of folates in RBCs, and consequently, the production of 5-methyl-THF leads to variations in cellular structures of one-carbon folate products. Accordingly, a plausible explanation for our results is that *MTHFR* 677 wild-type genotype individuals could have a precise effect on nucleotide synthesis by increasing the availability of 5,10-methylene-THF necessary for normal DNA synthesis and cell division.

Additionally, the A1298C *MTHFR* polymorphism is located in the region encoding the N-terminal catalytic domain; thus, individuals with the homozygous genotype for this variant do not have the enzymatic properties distinguishable from the wild type [34].

Meanwhile, the *MTHFR* C677T variant is located in the region encoding the C-terminal SAM regulatory domain of the enzyme. It has been suggested that *MTHFR* A1298C may act through a different pathway than *MTHFR* C677T [35, 36]. Based on this hypothesis, Krajinovic et al. [37] proposed that the variant A1298C tends to accumulate 5,10-methyl-THF, whereas the C677T variant tends to accumulate 5,10-methylene-THF. The accumulation of 5,10-methylene-THF might result in 5,10-methyl-THF/5,10-methylene-THF balance. We also agree and suggest that the amount of 5,10-methyl-THF, 5,10-methylene-THF, or 5,10-methyl-THF/5,10-methylene-THF could vary depending on each genotype of independent allelic variants or combined. According to this, we found that the combined genotypes of *MTHFR* 677CC+1298AC have a protective effect on leukemia (OR=0.23; 95% CI, 0.06–0.82; p=0.023, corresponding to a reduced risk of 4.3-fold with 95% CI, 1.2–16.7). Our results confirm and extend previous findings of Krajinovic et al. [37], who reported that the combined genotypes of *MTHFR* 677TT+1298AA and 677CC+1298CC were associated with a reduced risk of developing ALL.

Thus, it is noticeable that in their results, the protective effect occurs only when the combination of genotypes integrates the homozygous/wild-type or wild-type/homozygous genotypes in each case, with the OR values (OR=0.4; 95% CI, 0.2–0.9; and OR=0.3; 95% CI, 0.1–0.6; corresponding to a 2.5- and 3.3-fold reduced risk, respectively) being lower than the those reported in this study (4.3-fold). It can be said that the combination of wild-type/heterozygous (*MTHFR* 677CC+1298AC) reported in this study confers a greater protective effect on ALL than in the case of homozygous/wild-type (*MTHFR* 677TT+1298AA) or wild-type/homozygous (*MTHFR* 677CC+1298CC) genotypes reported in the study of Krajinovic et al. [37]. There may be two reasons for this outcome: first, it is possible that the folate amount in these genotype combinations is variable but sufficient to produce a correct thymidylate synthesis, which decreases uracil disincorporation [38]; second, the genetic variability in different populations [8, 9, 24, 25].

With respect to *TPMT*, the variants *TPMT**2 and *TPMT**3A were not implicated in genetic susceptibility to ALL; these data confirm and extend previous findings of Ouerhani et al. [14].

It is noticeable that a null association for the synergistic effect of both genes studied was found; however, future research in other populations will be necessary to further support our results. More importantly, to date, there are no reports focused on associations with synergistic effects of both genes and risk to ALL in children. There is only one report where the *MTHFR* 1298AC genotype appeared to reduce ALL risk in Chinese females and males, whereas the *MTHFR* 677TT and TS2R3R/2R2R genotypes increased ALL risk in Chinese adults with low folate intake [39]. There is also another study that shows lack of association for both genes and risk to sinusoidal obstruction syndrome in ALL patients with thioguanine exposure therapy [40]. In reference to dietary and environmental factors, low folate intake may modify ALL risk [28–30, 39] and coexistence of 677TT and 1298CC alleles may elevate toxicity risk by methotrexate treatment in pediatric ALL patients [41].

In conclusion, study and comprehension of the combination of genetic and environmental factors involved in folate-dependent enzyme activity is quite promising for treatment of childhood ALL patients. Therefore, our data suggest that the *MTHFR* 1298CC genotype is associated with susceptibility to childhood ALL and, at least in Mexicans, may represent a genetic marker for childhood ALL patients. Additionally, the presence of the *MTHFR* 677CC+1298AC haplotype confers increased protection (4.3-fold) against malignancy.

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