The methylenetetrahydrofolate reductase 677T-1298C haplotype is a risk factor for acute lymphoblastic leukemia in children

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Abstract
The etiology of acute lymphoblastic leukemia (ALL) is complex, linked with both environmental exposures and genetic factors. Functional variants of the methylenetetrahydrofolate reductase (MTHFR) gene result in disturbance in folate metabolism and may affect susceptibility to cancer. The study was performed to evaluate whether MTHFR C677T and A1298C polymorphisms, analyzed separately and together, are associated with the development of ALL in a population under 18 years of age of Caucasian ancestry.

The study included 117 pediatric patients (59% males, mean age at diagnosis 7.4 ± 6.2 years) with ALL, confirmed by conventional immunophenotyping surface-marker analysis and 404 healthy control subjects (48.5% men, mean age 37.7 ± 11.3 years). The MTHFR C677T and A1298C genotypes were analyzed using allele discrimination tests with Taq-Man fluorescent probes.

The MTHFR 677T genotype was related to a 2-fold increase in risk of ALL (P = .014). The 677T-1298C haplotype was found in ALL patients but not in controls (frequency 0.598%; P < .0001). The observed frequency of carriers of this rare haplotype was 12%, including 677CT/1298CC (1.7%), 677TT/1298AC (6.0%), and 677CT/1298AC (4.3%) genotypes.

The MTHFR 677T allele alone or in combination with the MTHFR 1298C allele significantly increases the risk of development of ALL in Polish population under 18 years of age. Further studies of haplotype composition in subjects with the 677CT/1298AC genotype are necessary to assess the risk of childhood ALL.

Abbreviations: 5,10-MTHF = 5,10-methyltetrahydrofolate, ALL = acute lymphoblastic leukemia, CI = 95% confidence interval, FAD = flavin adenine dinucleotide, GWAS = genome-wide association study, HWE = Hardy-Weinberg equilibrium, MTHFR = methylenetetrahydrofolate reductase, OR = odds ratio, P = probability, SAM = S-adenosylmethionine.

Keywords: acute lymphoblastic leukemia, ALL risk, MTHFR polymorphism

1. Introduction
Acute lymphoblastic leukemia (ALL) is a heterogeneous group of neoplasms which develop from rapidly proliferating malignant T or B lymphoid cells. It is the most frequently diagnosed malignancy in children and represents about 30% of all cancers diagnosed in this age group.[11] Peak incidence occurs between 2 and 5 years of age.[2]

The etiology of ALL is complex and not fully understood. It has been linked with both environmental exposures (including ionizing radiation, hydrocarbons, pesticides, and alcohol and cigarette use) and inherited genetic disorders (including Fanconi anemia, Down, and Bloom syndrome). However, these determinants are responsible for <10% of all ALL cases.[3] In children, the most important factors are thought to be interactions between genetic factors and fetal environmental exposures. A genome-wide association study has revealed a few susceptibility loci for childhood ALL, such as 9p21.3 (CDKN2A), 7p12.2 (IKZF1), 10q21.2 (ARIDB5), and 14q11.2 (CEBP).[4,5] However, knowledge of the genetic factors involved in ALL development is still limited, and needs to be expanded.

Methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) is an enzyme which plays a crucial role in folate metabolism. It is responsible for the irreversible conversion of 5,10-methyltetrahydrofolate (5,10-MTHF) into 5-MTHF, which is a cosubstrate for homocysteine to methionine remethylation.[6] The above-mentioned compounds are important for many cellular processes, such as DNA and RNA synthesis, DNA methylation and repair, and cysteine metabolism.[7] Thus, genetic variations in the gene encoding MTHFR may influence enzyme activity and, ultimately, affect susceptibility to cancerogenesis.[8] There are 2 common functional MTHFR gene (ENSG00000177000) polymorphisms, C677T (rs1801133) and A1298C (rs1801131), which result in
decreased enzyme activity. Literature data on the relationship between these genetic variants and ALL development are conflicting. In addition, there are few studies considering the distribution of MTHFR 677T/1298 genotypes and the 677-1298 haplotype in ALL patients. Therefore, the aim of this study was to evaluate whether MTHFR C677T and A1298C polymorphisms, analyzed separately and together, are associated with the development of ALL in children.

2. Materials and methods

2.1. Subjects
The study included 117 ALL pediatric patients: 48 females (41%) and 69 males (59%) of Caucasian ancestry, treated in the Department of Pediatric Oncology, Hematology and Bone Marrow Transplantation at the Poznan University of Medical Sciences, Poland. The mean age was 10.4 ± 5.5 years (range: 1–23 years), and mean age at diagnosis was 7.4 ± 5.2 years (range: 0.3–18 years). The ALL diagnosis was made according to the French-American-British criteria, after conventional immunophenotyping using surface-marker analysis. Inclusion criteria for the study group were diagnosis at under 18 years of age and absence of other malignancy.

The control group consisted of 404 healthy subjects without ALL: 208 females (51.5%) and 196 males (48.5%) with a mean age of 37.7 ± 11.3 years (age range 19–75 years), selected from an area of northwest Poland (the same part of Poland as for the selection of the ALL patients) in the years 2004 to 2012. Of this group, 93 subjects were selected randomly during the national health program for screening cardiovascular risk factors, and the rest were randomly selected couples whose children were born in hospitals in the Poznan district.

2.2. Ethics statement
The study was performed in accordance with the World Medical Association Declaration of Helsinki and approved by the Bioethics Committee of the Poznan University of Medical Sciences (resolution no. 1430/04, 1140/05, 1502/05, and 595/13).

2.3. Methods
Details of MTHFR C677T and A1298C genotyping have been described previously.[9] Briefly, DNA was extracted from 5 million cells after lysis of peripheral whole blood, using a DNA extraction kit (DNA Extraction Kit, Stratagene) in accordance with the manufacturer’s instructions. Next, the variants studied were identified using commercially available tests: MTHFR C677T and MTHFR A1298C kit (GeneProof) with fluorophore-labeled probes (Taq-Man hydrolysis probes).

2.4. Statistical analyses
Allele frequencies among patients were compared using the χ² test and Fisher exact test (GraphPad Software Inc, San Diego, CA, version 5.03). For risk assessment conferred by a particular variant, the values of the odds ratio (OR) were estimated with 95% confidence intervals (CI). Genotype data were analyzed under dominant, recessive, and additive models to maximize the power of detecting an association. Genotype frequencies were tested for Hardy–Weinberg equilibrium (HWE) using the χ² test (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl), while linkage disequilibrium between the MTHFR variants and haplotype frequencies was tested using Haplovew software.[10] The standardized linkage disequilibrium coefficient D’ (Lewontin’s D’D) was calculated. The differences were considered significant if the value of probability (P) did not exceed 0.05.

3. Results
To assess the association between MTHFR C677T and A1298C polymorphisms and ALL development, the frequencies of genotypes, alleles, and haplotypes from pediatric ALL patients (N=117) and the control group (N=404) were compared.

In all patients, the observed genotype frequencies for MTHFR C677T differed significantly from frequencies calculated for HWE (P = .006). Six MTHFR 677T/1298 genotype combinations (CC/AA, CC/AC, CC/CC, CT/AA, CT/AC, and TT/AA) were present in the control group, whereas 8 were present in all patients, including additional genotypes of carriers of both variant alleles (CT/CC and TT/AC; Table 1). The

| MTHFR polymorphisms | Genotype   | ALL patients N=117 | Control group N=404 | Univariate analysis, P |
|----------------------|------------|---------------------|----------------------|------------------------|
|                     |            |                     |                      |                        |
| C677T rs1801133      | CC         | 54 (46.2)           | 178 (44.1)           | 677TT, P=.0115*        |
|                     | CT         | 41 (35.0)           | 185 (45.8)           |                        |
|                     | TT         | 22 (18.8)           | 41 (10.1)            |                        |
|                     | P helpers  | 0.006               | 0.484                |                        |
| A1298C rs1801131     | AA         | 47 (40.2)           | 172 (42.6)           | NS                     |
|                     | AC         | 55 (47.0)           | 194 (48.0)           |                        |
|                     | CC         | 15 (12.8)           | 38 (9.4)             |                        |
|                     | P helpers  | 0.969               | 0.112                |                        |
| C677T/A1298C         | CC/CC      | 20 (17.1)           | 55 (13.0)            | 677TT/1298AC, P=.0001  |
|                     | CC/AC      | 21 (17.3)           | 85 (21.0)            |                        |
|                     | CC/CC      | 13 (11.1)           | 38 (9.4)             |                        |
|                     | CT/AA      | 12 (10.3)           | 76 (18.8)            |                        |
| rs1801133/rs1801131  | CT/AC      | 27 (23.1)           | 109 (27.0)           |                        |
|                     | CT/CC      | 2 (1.7)             | 0 (0.0)              | 677CC/1298CC + 677TT/1298AC, P=.0001 |
|                     | TT/AA      | 15 (12.8)           | 41 (10.1)            |                        |
|                     | TT/AC      | 7 (6.0)             | 0 (0.0)              |                        |

Variables are expressed as numbers (percentages).
ALL = acute lymphoblastic leukemia, HWE = Hardy–Weinberg equilibrium, MTHFR = methylenetetrahydrofolate reductase, NS = not statistically significant, P = probability value.
*OR = 2.1, 95%CI: 1.2–3.6.
Table 2

| MTHFR polymorphisms | Allele or haplotype | ALL patients N=117 | Control group N=404 |
|---------------------|---------------------|-------------------|-------------------|
| C677T rs1801133     | C                   | 149 (63.7)        | 541 (67.0)        |
|                     | T                   | 85 (33.6)         | 267 (33.0)        |
| A1298C rs1801131    | A                   | 149 (63.7)        | 538 (66.6)        |
|                     | C                   | 85 (33.6)         | 270 (33.4)        |
| C677T-A1298C        | C-A                 | 78 (33.3)         | 271 (33.3)        |
|                     | C-C                 | 71 (30.3)         | 270 (33.4)        |
| n1801133/rs1801131  | T-A                 | 71 (30.3)         | 267 (33.0)        |
|                     | T-C                 | 14 (5.9)          | 0 (0.0)           |

Variables are expressed as numbers (percentages).
ALL = acute lymphoblastic leukemia, MTHFR = methylenetetrahydrofolate reductase. *P < .0001.

Despite many studies on the etiology of ALL, the causal mechanism underlying disease development is still unspecified. Recently, there has been growing interest in the significance of folate metabolism in modifying the risk of cancerogenesis. Much attention has been paid to the enzymes involved in the folate pathway, including MTHFR. It is known that genetic variance in MTHFR may affect enzyme activity and, in consequence, disturb folate metabolism, DNA synthesis, and methylation. These disturbances may lead to increased cancer susceptibility.\(^{11}\)

The gene encoding MTHFR is located on the short arm of chromosome 1 (1p36.3), consists of 11 exons, and comprises a region of >20,000 bp.\(^{12}\) There are 2 common functional MTHFR polymorphisms, C677T and A1298C, which influence enzyme activity. C>T transition occurs at nucleotide 677 in exon 4 of the MTHFR gene, where the flavin adenine dinucleotide (FAD)-binding site is located. It causes an alanine to valine amino acid substitution (Ala222Val), which results in impaired FAD binding, enzyme thermolability, and decreased activity.\(^{13-15}\) It has been shown that 677TT homozygotes and 677CT heterozygotes have about 70% and 40% reduced MTHFR activity, respectively.\(^{11,16}\) Moreover, the 677TT genotype has been found to be associated with a lower level of genomic DNA methylation.\(^{17}\) In turn, A>C transition at nucleotide 1298 occurs in exon 7 within the S-adenosylmethionine-binding domain and results in the substitution of glutamic acid for alanine (Glu429Ala).\(^{18}\) 1298CC homozygotes have about 20% reduced MTHFR activity. The exact mechanism underlying the influence of A1298C on MTHFR activity and the functional consequences have not been fully elucidated. Some studies show no association with enzyme thermolability or homocysteine concentration.\(^{19,20}\) However, it was shown that coexistence of the 2 variant alleles, that is, 677TT and 1298CC, results in about 60% to 70% reduction of MTHFR activity, which is comparable to the effect observed in the 677TT homozygotes.\(^{21}\)

MTHFR C677T and A1298C polymorphisms have been studied extensively in the context of many human diseases and conditions, such as: lung cancer,\(^{22}\) cervical cancer,\(^{23}\) rheumatoid arthritis,\(^{24}\) myocardial infarction,\(^{25}\) migraine,\(^{26}\) and recurrent pregnancy loss.\(^{27}\) There are also studies on the association between the occurrence of MTHFR polymorphisms and ALL development in adults and children in different regions and subpopulations. However, data derived from these studies are inconsistent, and very often even conflicting.\(^{6,28-31}\) which makes the significance of the MTHFR gene in the risk of childhood ALL unclear. Moreover, in some studies, only one variant is analyzed, prevalently, the C677T polymorphism, or, despite both polymorphisms being genotyped, they are analyzed separately. In our study, we assessed MTHFR C677T and A1298C genotypes, allele, and haplotype frequencies. The minor allele frequencies observed in the groups studied were comparable with those for a European subpopulation, which, according to the e! EnsemblGenomes 32 database (released August 4, 2016), were 0.36 for the 677TT allele and 0.31 for the 1298C allele. In our study, the respective values were 0.36 and 0.33, and did not differ significantly between the ALL group and the control group. In an analysis that takes into account the effects of a single variant, we found that the 677TT genotype was significantly associated with a 2-fold higher risk of ALL development (a recessive effect of the 677TT allele, P = .014). The separate effect of the A1298C variant was not statistically significant; however, in combination with the 677TT allele (677TT-1298C haplotype), it had a significant influence on ALL risk. Moreover, the association of the 677TT-1298C
haplotype with disease development was much stronger \((P < 0.0001)\) than was observed for the \(677T\) allele alone.

In a meta-analysis Li et al.\(^{[12]}\) investigated the association between \(MTHFR\) \(677T\) and ALL risk in children of Caucasian ancestry, and did not find a significant association in any of the five models analyzed (allele contrast, additive, recessive, dominant, and effects of heterozygous genotype).\(^{[12]}\) Amigou et al.\(^{[28]}\) also found no association between both \(677T\) and \(1298C\) and ALL risk when analyzing a group of 648 children with ALL and 1681 healthy controls. In turn, Lightfoot et al.\(^{[6]}\) analyzing these variants in 939 children with ALL and 824 controls in the United Kingdom, did not observe a significant association for \(677T\), but observed that the \(1298C\) variant is associated with slightly lower risk of ALL \((OR=0.79, P=0.02)\). On the other hand, De Jonge et al.\(^{[31]}\) and Chatzidakis and Goulas\(^{[34]}\) observed a protective effect of allele \(677T\) in regard to ALL in children, with OR=0.7 and OR=0.4, respectively. De Jonge et al.\(^{[31]}\) also analyzed \(A1298C\) but did not find a significant difference between groups.

One explanation of the inconsistency between the results of various researchers may be the different levels of folates in the populations studied, as well as differences in the selection of patients. It was demonstrated that the association between \(MTHFR\) polymorphisms and ALL risk in children can be modified by supplementation of folic acid before as well as during pregnancy. Krajnovic et al.\(^{[35]}\) analyzed \(MTHFR\) variants in a group of 270 ALL children and 300 healthy controls of French–Canadian origin. The group of ALL patients consisted of those born before as well as after the introduction of mandatory maternal folate supplementation during pregnancy. Only in children born before the introduction of folate supplementation was the protective effect of the \(677T\) and \(1298C\) allele found. The authors suggest that the effect of \(MTHFR\) polymorphisms is less relevant when the folate level is adequate.\(^{[35]}\)

As mentioned above, the carriers of the variant alleles of both the \(MTHFR\) polymorphisms analyzed have decreased enzyme activity up to 30% to 40%. Therefore, it seems reasonable to analyze both variants and genotype combinations. In our study, we observed 8 different \(677T/1298\) genotypes, including 2 which were found only in ALL patients and not in the controls: \(677CT/1298CC\) and \(677TT/1298AC\). Similarly, an additional \(677T\)–\(1298C\) haplotype was only found in the ALL group, and did not occur in healthy subjects. This suggests that the frequency of occurrence of crossing over during the formation of gametes in parents of children suffering from ALL might be higher than observed in the normal population. The inheritance of rare disadvantageous DNA variants resulting from crossing over in leukemia predisposing pathways that could affect overall genomic instability was previously found in the case of Fanconi anemia genes.\(^{[36]}\) In contrast, there was no difference in the distribution between the groups studied for other common \(MTHFR\) \(677T/1298\) genotypes.

A study by Goricar et al.\(^{[31]}\) found a relationship between the \(677T/1298A\) haplotype and a decreased risk of ALL in pediatric patients \((OR=0.65, P=0.03)\). The authors reported only three \(677–1298\) haplotypes in the groups studied, \(C–A\), \(C–C\), and \(T–A\), and their frequencies are consistent with our results. To the best of our knowledge, most studies on \(MTHFR\) polymorphisms in childhood ALL do not analyze haplotypes. If haplotypes are analyzed, only the 3 mentioned above are found. However, based on literature data on \(MTHFR\) \(677T/1298\) distribution, we noticed that genotypes \(CT/AC\), \(CT/CC\), and \(TT/AC\), as well as the \(T–C\) haplotype were found in different diseases and conditions with frequencies similar to those observed in our ALL patients. Dekou et al.\(^{[37]}\) observed a \(CT/AC\) genotype in 19% of a British population sample \((23.1%\) in our ALL patients), \(CT/CC\) in 1.4% \((1.7%\) in our ALL patients), and \(TT/AC\) in 1.8% \((6.0%\) in our ALL patients). Isotalo and Donnelly\(^{[38]}\) found the \(CT/AC\) genotype in 32% of patients with venous thrombosis and 53% of healthy volunteers, and \(TT/AC\) in 9.2% patients and 13% in controls. The higher frequency of the \(677T/1298C\) haplotype in these populations may be a result of the founder effect. Data collected by Ogino and Wilson\(^{[39]}\) revealed that the genotype \(TT/CC\) has also been found in some studies. \(MTHFR\) \(677T/1298\) genotype frequencies estimated by the authors in the general population are 0.21% for \(CT/CC\) and 0.22% for \(TT/AC\).\(^{[39]}\)

This study has some limitations. The first is the ALL sample size. However, because the main results are based on genotypes that were not observed in the controls, the sample was large enough to obtain highly significant results. Moreover, this study includes a relatively large control sample. A second limitation was that we could not distinguish haplotypes in the compound heterozygote \(677CT/1298AC\).

Based on the results obtained, it can be summarized that in the Polish pediatric population, carriers of the \(MTHFR\) \(677T/1298C\) haplotype (i.e. subjects with \(677CT/1298CC\) and \(677TT/1298AC\) genotypes) have a 2-fold higher risk of ALL development. As carriers of \(677T/1298C\) were not present in the control group, and homozygous carriers of the \(677T\) allele were also observed in >10% of the control group, it might be suggested that both variants should be analyzed in order to estimate the risk of ALL in pediatric patients. These variants are functional, they may be considered as causal risk factors of ALL. To confirm our finding, additional studies with larger sample sizes are necessary. Further studies of the composition of the haplotype in compound \(677CT/1298AC\) heterozygotes are crucial to ALL risk assessment. Moreover, there is also a need for further studies on the biological role of both polymorphisms in ALL patients and their parents in order to determine their influence on the pathomechanisms of ALL development.
Barrett JC, Fry B, Maller J, et al. Haplview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21:263–5.

Kim YI. Role of the MTHFR polymorphisms in cancer risk modification and treatment. Future Oncol 2009;5:523–42.

Gaughan DJ, Barbaxu S, Kluittmans LA, et al. The human and mouse methylenetetrahydrofolate reductase (MTHFR) genes: genomic organization, mRNA structure and linkage to the CLCN6 gene. Gene 2000;257:279–89.

Frost P, Blom HJ, Milos R, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet 1995;10:111–3.

Guenter BD, Sheppard CA, Tran P, et al. The structure and properties of methylenetetrahydrofolate reductase from Escherichia coli suggest how folate ameliorates human hyperhomocysteinemia. Nat Struct Mol Biol 1999;6:359–65.

Cohen-Woods S, Craig I, Gaysina D, et al. The Bipolar Association Case-Control Study (BACCS) and meta-analysis: no association with the 5,10-methylenetetrahydrofolate reductase gene and bipolar disorder. Am J Med Genet B Neuropsychiatr Genet 2010;153B:1298–304.

Rozen R. Molecular genetics of methylenetetrahydrofolate reductase deficiency. J Inherit Metab Dis 1996;19:589–94.

Castro R, Rivera I, Ravasco P, et al. 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C>T and 1298A>C mutations are associated with DNA hypomethylation. J Med Genet 2004;41:454–8.

Van der Put N, Bagreels F, Tran P, et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor neural-tube defects. Am J Hum Genet 1999;65:1044–51.

Hanson N, Aras O, Yang F, et al. C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase gene: incidence and effect of combined genotypes on plasma fasting and post-methionine load homocysteine in vascular disease. Clin Chem 2001;47:661–6.

Friso S, Girelli D, Trabetti E, et al. A1298C methylenetetrahydrofolate reductase mutation and coronary artery disease: relationship with C677T polymorphisms and homocysteine/folate metabolism. Clin Exp Med 2002;2:7–12.

Brockton N. Localized depletion: the key to colorectal cancer risk mediated by MTHFR genotype and folate? Cancer Causes Control 2006;17:1005–16.

Yang Y, Yang LJ, Deng MZ, et al. MTHFR C677T and A1298C polymorphisms and risk of lung cancer: a comprehensive evaluation. Genet Mol Res 2016;15: DOI: 10.4238/gmr.15027615.

Yi K, Yang L, Lan Z, et al. The association between MTHFR polymorphisms and cervical cancer risk: a system review and meta-analysis. Arch Gynecol Obstet 2016;294:579–88.

Cen H, Huang H, Zhang LN, et al. Associations of methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C polymorphisms with genetic susceptibility to rheumatoid arthritis: a meta-analysis. Clin Rheumatol 2016;36:287–97.

Alizadeh S, Djafarian K, Moradi S, et al. C677T and A1298C polymorphisms of methylenetetrahydrofolate reductase gene and susceptibility to myocardial infarction: a systematic review and meta-analysis. Int J Cardiol 2016;217:99–108.

Kara I, Sazci A, Ergul E, et al. Association of the C677T and A1298C polymorphisms in the 5,10-methylenetetrahydrofolate reductase gene in patients with migraine risk. Brain Res Mol Brain Res 2003;111:84–90.

Rai V. Methylenetetrahydrofolate reductase C677T polymorphism and recurrent pregnancy loss risk in Asian population: a meta-analysis. Ind J Clin Biochem 2016;31:402–13.

Metayer C, SkelO G, Chokkalingam AP, et al. Genetic variants in the folate pathway and risk of childhood acute lymphoblastic leukemia. Cancer Causes Control 2011;22:1243–58.

Amigou A, Rudant J, Orsi L, et al. Folic acid supplementation, MTHFR and MTRR polymorphisms, and the risk of childhood leukemia: the ESQUARE study (SFCE). Cancer Causes Control 2012;23:1265–77.

Wang H, Wang J, Zhao L, et al. Methylenetetrahydrofolate reductase polymorphisms and risk of acute lymphoblastic leukaemia - evidence from updated meta-analysis including 35 studies. BMC Med Genet 2012;13:77.