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Methylenetetrahydrofolate reductase genotypes do not play a role in acute lymphoblastic leukemia pathogenesis in the Italian population

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A B S T R A C T

Background and Objectives. Methylenetetrahydrofolate reductase (MTHFR) is one of the enzymes involved in folate metabolism and DNA methylation and synthesis. Some genotypes of this highly polymorphic enzyme are associated with decreased activity. Previous studies have suggested that individuals with the MTHFR 677T, 1298AC and 1298CC have a lower risk of adult acute lymphoblastic leukemia (ALL).

Design and Methods. In order to test this association we studied the presence of the C677T and A1298C mutant alleles in 174 patients with acute lymphoblastic leukemia and in 110 controls from central Italy.

Results. We did not find any association between the different polymorphisms and susceptibility to ALL. In multivariate analysis different genotypes did not show any correlation with the risk of ALL. The adjusted odds ratios and 95% confidence intervals for MTHFR C677T were 0.69 (0.4-1.19) for 677CT versus 677CC wild type, and 0.99 (0.50-1.97) for 677TT versus 677CC. The corresponding values for MTHFR A1298C were 0.93 (0.56-1.53) for 1298AC versus 1298AA wild type and 1.14 (0.36-3.61) for 1298CC versus 1298AA.

Interpretations and Conclusions. These results do not support the suggestion that populations carrying different genotypes of the two MTHFR polymorphisms, C677T and A1298C, have a different susceptibility to ALL, at least in the Mediterranean area.

Key words: MTHFR gene, DNA repair, acute leukemia susceptibility.

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Methylenetetrahydrofolate reductase (MTHFR) has a crucial role in regulating cellular methylation, through the conversion of 5,10-methyl-tetrahydrofolate (THF) to 5-methyl-THF, the methyl donor in the transformation of homocysteine to methionine.^{1,2} The common C-T polymorphism at nucleotide 677 (C677T), which results into an alanine to valine substitution in the MTHFR protein, causes higher thermolability and reduced enzyme activity.³ Increased folic acid intake is required to maintain homocysteine remethylation to methionine at normal levels (Figure 1). Reduced MTHFR activity due to the C677T change has been associated with different disorders, including vascular disease, neural tube defects, Down's syndrome, eclampsia and inflammatory bowel diseases.⁴⁻⁸ Moreover, various authors have studied the association of this polymorphism with the risk of

colon, ovarian, breast, lung and esophageal squamous cell cancer.⁹⁻¹³ The suggested lower risk of colon cancer in individuals carrying at least one T allele is thought to be caused by more reliable DNA synthesis because of a greater availability of 5,10-methylene tetrahydrofolate for DNA synthesis.^{9,10} However, studies performed in different populations have often shown inconsistent results and controversies about these associations have been raised.

A second mutation A1298C (glutamate to alanine) has been described. This mutation has been associated with increased levels of homocysteine in people carrying the 677CT and 677TT polymorphisms. In fact heterozygotes for both the C677T and the A1298C mutations had 50-60% of normal enzyme activity, a value which was lower than that seen in individuals who were heterozygous for the C677T variant.

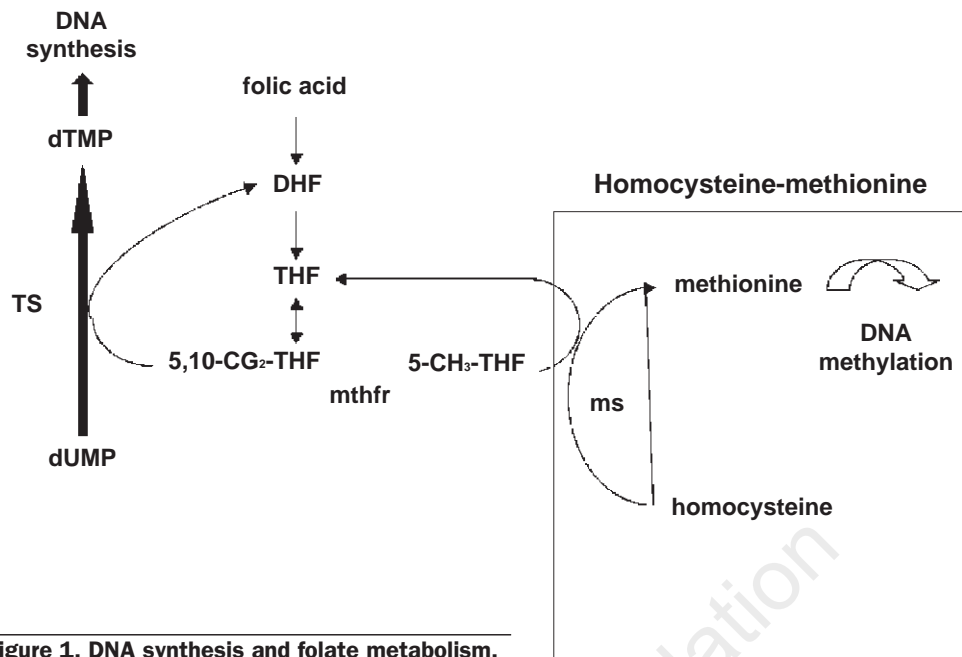


Figure 1. DNA synthesis and folate metabolism.

No individuals have been found to be homozygous for both mutations.^{14,15}

Recently, both the C677T and A1298C polymorphisms have been related to a variable risk of acute lymphoblastic leukemia (ALL).¹⁶

The hypothetical effect of C677T and A1298C in lowering the risk of ALL is explained like that for colon cancer, by reduced misincorporation of uracil instead of thymidine in DNA strands because of the greater availability of methyl donors as a result of reduced activity of MTHFR.

In order to verify the association between the MTHFR genotype and the risk of ALL, we investigated the prevalence of the C677T and A1298C polymorphisms in a large cohort of Italian ALL patients enrolled in the GIMEMA (Gruppo Italiano Malattie EMatologiche dell'Adulto) multicenter studies ALLO288, ALLO496, ALLO597 and ALL2000 and compared the results to those recorded in control subjects.

Design and Methods

One hundred and seventy-four patients affected by ALL and 110 controls entered the study. All 174 patients were analyzed through central handling of the samples at presentation, which were investigated for morphology, immunophenotype, cytogenetics, molecular biology and multidrug resistance; these analyses are part of the work-up for all adult ALL patients entering GIMEMA trials. All patients and controls were Caucasians from central Italy. Patients' samples were collected from the centralized laboratory of

the GIMEMA study group from 1995 and those of the healthy individuals from the Blood Donor Center at the Catholic University Hospital in Rome. Donors were excluded if they had a family history of hematologic diseases. The median age of cases was 32 years (range 5–75) and that of controls was 37 years (range 20–66). The group of patients was formed of 96 males (55.1%) and 78 females; the healthy control group comprised 75 males (68%) and 35 females.

MTHFR C677T genotypes were analyzed on DNA from peripheral blood cells with a method adapted from Frosst *et al.*¹⁷ Briefly, 1 µg of DNA was amplified with 100 ng of the specific primers. The 198bp polymerase chain reaction (PCR) products were digested with HinfI restriction enzyme for 2 hours at 37°C. After digestion, samples were run on a 4% agarose gel. The wild type genotype (677CC) produces a single band at 198bp, heterozygotes (677CT) produce 198bp, 175bp and 23bp fragments and homozygotes (677TT) produce 175bp and 23bp fragments.

MTHFR A1298C genotypes were analyzed on DNA (1 mg) from peripheral blood cells by PCR amplification of a 145bp fragment¹⁴ with 100 ng of specific primers. The 163bp PCR products were digested with MboII restriction enzyme for 2 hours at 37°C. After digestion, samples were run on a 30% polyacrylamide gel. The wild type genotype (1298AA) produces five bands of 56, 31, 30, 28, and 18bp, heterozygotes (1298AC) produce six bands of 84, 56, 31, 30, 28, and 18bp, and homozygotes (1298CC) produce four fragments of 84, 31, 30 and 18bp.

Statistical analysis

Statistical analysis was performed using STATA 6.0™ (STATA, College Station, TX, USA) software. Pearson's χ^2 was used to compare differences in proportions. The risk of people with different genotypes developing the disease was measured as an odds' ratio. People with the wild type genotypes (677CC and 1298AA) were considered at baseline risk. Multiple logistic regression was performed, including sex and age as covariates in the model. A p value <0.05 is considered statistically significant. The 95% confidence intervals are also given.

Results

The distribution of the MTHFR C677T and A1298C genotypes in cases and controls are summarized in Table 1. We found the MTHFR 677CC genotype in 65 cases (37.3%) and 35 controls (31.8%), the 677CT genotype in 71 cases (40.8%) and 55 controls (50%) and the 677TT genotype in 38 cases (21.8%) and 20 controls (18.1%). For MTHFR 1298, we observed the 1298AA genotype in 92 cases (52.8%) and 56 controls (50.9%), the 1298AC genotype in 73 cases (41.9%) and 49 controls (44.5%) and the 1298CC genotype in 9 cases (5.1%) and 5 controls (4.5%). The frequencies of the 677T allele and 677TT genotype were 0.42 and 0.21, respectively, in patients, and were very similar to the values in the control group (0.43 and 0.18, respectively). We observed similar 1298C allele and 1298CC frequencies in cases and controls (0.26 and 0.05 for cases and 0.27 and 0.04 for controls, respectively). The distribution of the genotypes among the controls was in Hardy-Weinberg equilibrium. There were no statistically significant differences in genotype distribution between cases and controls ($p = 0.3$ for C677T and $p = 0.9$ for A1298C). We did not find any double homozygotes for both the polymorphisms (677TT and 1298CC) suggesting that having these two mutations together may be fatal during embryonic development. Univariate analysis in a logistic regression model did not show any statistical correlation between the variant MTHFR genotypes, 677CT and 677TT, and protection from the risk of ALL, when this risk was compared with that of subjects with the 677 wild type genotype (OR 0.70, 95% CI 0.41–1.19 for 677CT and OR 1.02, 95% CI 0.52–2.01 for 677TT) ($p = 0.18$ and $p = 0.94$, respectively). Similarly, there was no significant association between the MTHFR A1298C genotype and susceptibility to ALL (OR 0.90, 95%CI 0.55–1.48 for 1298AC and OR 1.09, 95%CI 0.34–3.43 for 1298 CC) ($p = 0.69$ and $p = 0.87$, respectively).

Even in multivariate models including sex and age as covariates, the different genotypes did not show any correlation with a lower risk of leukemia (OR 0.74 95%

Table 1. Genotype distributions of MTHFR 677 and 1298 polymorphisms.

MTHFR 677			
Genotype	CC (%)	CT (%)	TT (%)
Cases (n=174)	65 (37.4%)	71 (40.8%)	38 (21.8%)
Controls (n=110)	35 (31.8%)	55 (50%)	20 (18.2%)
MTHFR 1298			
Genotype	AA (%)	AC (%)	CC (%)
Cases (n=174)	92 (52.9%)	73 (41.9%)	9 (5.2%)
Controls (n=110)	56 (50.9%)	49 (44.5%)	5 (4.6%)

CI 0.43–1.29 for 677CT and OR 1.04, 95% CI 0.51–2.09 for 677TT) ($p = 0.29$ and $p = 0.89$ respectively) (OR 0.91 95% CI 0.55–1.49 for 1298AC and OR 1.27 95% CI 0.41–4.05 for 1298CC) ($p = 0.70$ and $p = 0.68$ respectively) (Table 2 and 3).

We also investigated the combined effects of the two polymorphisms in a bivariate model but we did not find any reduction in the risk of patients with such combinations developing ALL in comparison with the risk in the reference group with 677CC/1298AA.

Discussion

The most common mutation of MTHFR is a C-T substitution at bp 677 which causes a substitution of valine for alanine in the enzyme.¹⁸ This single substitution can be detected functionally because it reduces the stability of the enzyme during *in vitro* incubation of cell extracts at 46°C for 5 minutes. This is an autosomal recessive mutation. Individuals who are homozygotes for the mutation have lower specific activity of MTHFR and the enzyme is less stable *in vitro*; hence the term *thermolabile MTHFR*. The frequency of the C677T polymorphism of MTHFR varies among racial and ethnic groups. Analysis of Caucasian and Asian populations shows rates from 12 to 19% for homozygotes (T/T) and up to 50% for heterozygotes (C/T). The incidence of the T/T genotype is very low among African-Americans whereas there are substantial variations among Caucasians.¹⁹ Rosenberg *et al.*¹⁹ considered that adequate folic acid intake in a given population may give rise to an increase in the frequency of the MTHFR 677T allele, whereas an inadequate intake may result in a decreased frequency. In this regard, the north to south increase in the prevalence of the MTHFR 677T allele in Europe may be influenced by the

Table 2. Odds ratios and 95% confidence intervals (95% CI) for MTHFR 677 and 1298 genotypes.

Genotype	Cases (n=174)	Controls (n=110)	OR	95%CI	p
MTHFR 677					
CC	65 (37.4%)	35 (31.8%)	1	–	
CT	71 (40.8%)	55 (50%)	0.70	0.41-1.19	0.18
TT	38 (21.8%)	20 (18.2%)	1.02	0.52-2.01	0.94
MTHFR 1298					
AA	92 (52.9%)	56 (50.9%)	1	–	
AC	73 (41.9%)	49 (44.5%)	0.90	0.55-1.48	0.69
CC	9 (5.2%)	5 (4.6%)	1.09	0.34-3.43	0.87

Table 3. Odds ratios and 95% confidence intervals (95% CI) for MTHFR 677 and 1298 genotypes in multivariate analysis.

	Adjusted OR (age included as covariate)	Adjusted OR (age and sex included as covariates)
C677T		
CC	1	1
CT	0.69 (0.40-1.19) p = 0.18	0.74 (0.43-1.29) p = 0.29
TT	0.99 (0.50-1.97) p = 0.99	1.04 (0.53-2.09) p = 0.89
A1298C		
AA	1	1
AC	0.93 (0.56-1.53) p = 0.78	0.91 (0.55-1.49) p = 0.70
CC	1.14 (0.36-3.61) p = 0.81	1.27 (0.41-4.05) p = 0.68

higher folic acid content in the diet of Mediterranean populations than in the diet of Northern European populations.²⁰

Chen *et al.* found a significant inverse correlation (i.e. a protective effect) between the T/T genotype for MTHFR and the risk of colorectal cancer in a case-control study.¹⁰ Men with the T/T genotype and normal plasma folate concentrations had a threefold lower risk of cancer than did men with either the C/C or C/T genotype. In contrast, men with the T/T genotype who were folate deficient had a risk of colorectal cancer that was comparable to that of subjects with the other genotypes. A possible explanation for this phenomenon is that reduced activity of the thermolabile MTHFR variant could have a positive effect on nucleotide synthesis by increasing the availability of 5,10-methylene-THF required for normal DNA synthesis and cell division. Chen *et al.* hypothesized that cells from folate-replete individuals with the T/T genotype may be less prone to insuf-

ficiencies in the pools of nucleotide precursors available for DNA synthesis. Folate deficiency has been shown to depress thymidylate synthesis, which increase the misincorporation of uracil and increases the frequency of chromosome breaks in human leukocyte DNA.

A second common polymorphism of MTHFR has been shown to involve an A-C substitution at bp 1298, which causes a Glu-Ala substitution in the MTHFR protein. The allele frequency of this substitution is similar to that of the C677T mutation.¹⁵ The significance of this polymorphism, if any, requires further investigation. Its role is probably related to the presence of a heterozygote or homozygote status of C677T.

Recently, Skibola *et al.* reported an association between susceptibility to adult ALL and polymorphisms in the gene encoding the MTHFR enzyme. In particular, they showed that individuals carrying at least one MTHFR mutation at the 677 (C→T) or 1298 (A→C) had a lower risk of developing ALL.¹⁶

Wiemels *et al.* reported associations of MTHFR polymorphisms in different subgroups of pediatric leukemias.²¹ The genotypes CT and TT at nucleotide 677 were shown to have a significant protective effect against MLL⁺ leukemia (OR=0.26 and 0.53 for heterozygotes and homozygotes, respectively). In contrast there was no evidence of a protective effect for the A1298C variant. A significant association was present for TEL-AML1 leukemias in which AC and CC genotypes at 1298 were overrepresented in the controls vs among patients with leukemia. A significant protective effect of the A1298C variant in its homozygous form was apparent for hyperdiploid leukemias. Franco *et al.* observed a protective effect only for the 677TT variant in childhood.²²

The above data contrast with those obtained in our study, which do not demonstrate a decreased risk of ALL in Italian adult patients with the T allele at 677 and the C allele at 1298. In our study the frequencies of the variant MTHFR 677TT genotype and the 677T allele were

0.21 and 0.42, respectively, and those of the MTHFR 1298CC genotype and 1298 C allele were 0.05 and 0.26 which were consistent with those among the controls. The lack of association between the MTHFR C677T and A1298C genotypes and a reduced risk of ALL may be influenced by the higher prevalence of 677T allele in the Italian population than in the Northern European population and by the apparently higher folic acid content in the diet of Mediterranean populations than in that of northern countries. Thus, the variant genotypes did not have any effect on ALL susceptibility, suggesting that other molecular mechanisms such as DNA damage and repair may play a major role in the etiology of ALL in this large sample population of Italians.

As recently suggested by Krajinovic *et al.*,²³ who provided additional evidence on the protective role of MTHFR polymorphisms in pediatric acute lymphoblastic leukemia, the effect of MTHFR polymorphism is less apparent when folate levels are adequate and the predictive importance of MTHFR polymorphisms in leukemia susceptibility would be of less relevance in subjects receiving a folate supplemented diet.

However, we cannot exclude alternative explanations to account for the observed difference, such as the lower power of our study in respect to that of other published studies or a different etiology of adult vs childhood leukemia. Indeed, our series included only five children.

These data confirm previous studies showing a high prevalence of the T allele in the Italian population.^{24,25} The present results do not rule out a protective effect of the T allele on ALL risk, but suggest that in populations with an adequate dietary intake of folate the reduced enzymatic activity of MTHFR in carriers of the T allele is balanced by folate uptake. This coincides with the observation that, despite a higher prevalence of 677T in Italy than in other European countries, the prevalences of cardiovascular disease, intestinal bowel diseases, neural tube defects and Down's syndrome are not increased in the Italian population.²⁶⁻³⁰ Papa *et al.*²⁴ described no significant difference in the prevalence of homozygotes for the 677TT variant of MTHFR between patients with intestinal bowel disease and controls. De Stefano *et al.*²⁵ concluded that homozygous MTHFR TT is highly frequent in the Italian population but that it is not associated with an increased risk for venous thrombosis. Regarding Down's syndrome, Stuppia *et al.*²⁶

confirmed the high prevalence of the T allele in the Italian population: they did not suggest an effect of the 677T allele on maternal non-disjunction, but rather suggested that in populations with an adequate dietary intake of folate the reduced enzymatic activity of MTHFR in carriers of the T allele is balanced by dietary folates. Abbate *et al.*²⁷ found no excess of homozygotes for the C677T polymorphism among patients with coronary artery disease with respect to among healthy subjects (28.5% vs 30.2%); moreover, the mutated genotype was not found to be related to the clinical manifestations of coronary artery disease or to the rate of restenosis after percutaneous transluminal coronary angioplasty. D'Elia *et al.*²⁸ found similar frequencies of C677T mutations among pre-eclamptic women and women with normal pregnancies.

De Franchis *et al.*²⁹ reported a lower incidence of spina bifida in Italy than in Northern European countries, again probably because of a higher intake of folic acid which could protect against diseases associated with the homozygous MTHFR 677 TT genotype. In a comparative studies of frequencies of candidate gene polymorphisms in cardiovascular disease in a French and Italian populations, Pallaud *et al.*³⁰ found that the allele frequency of MTHFR 677T differed between the two European countries suggesting the existence of a north-south gradient in allele frequency in Europe. In conclusion, our study does not provide any evidence of an association between the MTHFR C677T and A1298C variant genotypes and a susceptibility to ALL. Thus, genotyping the MTHFR gene does not appear to be useful for identifying individuals at different risks of developing ALL. We are currently investigating other polymorphisms involved in folic acid metabolism (thymidylate synthase, serine-hydroxymethyltransferase and methionine synthase) which could be involved in the pathogenesis of ALL and could influence treatment outcome and tolerance of the treatment itself.^{31,32}

PC designed the study with GC and SS; AV was responsible for DNA extraction; PC, GR, AF and GF were responsible for molecular test of both MTHFR polymorphisms; PF, LL and FS collected the data on patients; FB was responsible for the statistical analysis; FM and GL reviewed the manuscript and gave final approval. The authors indicated no potential conflicts of interest.

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