The *MTHFR 1298A>C* Polymorphism and Genomic DNA Methylation in Human Lymphocytes

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Abstract

Methylenetetrahydrofolate reductase (MTHFR) balances the pool of folate coenzymes in one-carbon metabolism for DNA synthesis and methylation, both implicated in carcinogenesis. Epidemiologic studies have shown that two functional polymorphisms in *MTHFR* gene, *677C>T* and *1298A>C*, are related to increased cancer risk. We aimed to analyze lymphocyte DNA from 198 subjects to evaluate the *MTHFR 1298A>C* polymorphism and folate status affecting genomic DNA methylation as a possible mechanism underlying the relationship between *MTHFR* polymorphisms and cancer susceptibility. Carriers of the *1298AA* wild-type genotype showed lower genomic DNA methylation compared with *1298AC* or *1298CC* genotypes [3.72 versus 8.59 or 6.79 ng 5-methyl-2′-deoxycytidine (5-mCyt)/μg DNA, $P < 0.0001$ and $P = 0.007$, respectively]. When DNA methylation was evaluated according to plasma folate status, only *1298AA* with low folate levels revealed diminished DNA methylation ($P < 0.0001$). Moreover, when the two *MTHFR* polymorphisms were concomitantly evaluated at the low folate status, DNA methylation was reduced only in *1298AA/677TT* compared with *1298AA/677CC* (3.11 versus 7.29 ng 5-mCyt/μg DNA, $P = 0.001$) and *1298CC/677CC* genotypes (3.11 versus 7.14 ng 5-mCyt/μg DNA, $P = 0.004$). However, the high prevalence of *677TT* mutants within the *1298AA* group (79%) and the similar biochemical features of *1298AA/677CC* and *1298CC/677CC* combined genotypes suggest that the gene-nutrient interaction affecting DNA methylation in *1298AA* is mainly due to the coexistence of the *677TT* genotype and that the *1298A>C* polymorphism may convey its protective effect not through this interaction but through another pathway in one-carbon metabolism. Further mechanistic studies are warranted to investigate how single polymorphisms as well as *MTHFR* combined genotypes exert their effect on cancer susceptibility. (Cancer Epidemiol Biomarkers Prev 2005;14(4):938–43)

Introduction

The altered properties of lymphoid neoplastic cells have been traditionally attributed to changes in the gene sequence or structure such as point mutations, gene deletions/rearrangements, or double strand breaks with subsequent increased risk of chromosomal translocations. Epigenetic abnormalities, widely described in several solid tumor malignancies, have been similarly found in hematopoietic cancer cells, suggesting that epigenetic phenomena can play a substantial role also in the carcinogenetic process affecting this particular tissue (1). DNA methylation is the most important epigenetic feature of eukaryotic DNA for the regulation of gene expression and the maintenance of genome integrity (2, 3). Preserving the patterns of DNA methylation is a major feature of DNA for the epigenetic control of genome stability and the regulation of gene transcription (4). An altered profile of DNA methylation is a frequent finding in cancer diseases, indicating that an aberrant regulation of the mechanisms of DNA methylation is crucial for the understanding of mechanisms of carcinogenesis (1, 5) and may constitute a good target for future therapies (5, 6). Abnormal genomewide DNA methylation is a phenomenon known to lead to chromosome instability and damage (7), genetic abnormalities that are frequently described in malignancies, mostly those of the lymphoid tissue.

A crucial role for the control of DNA methylation as well as DNA synthesis and repair mechanisms is played by the provision of adequate pools of methyl groups through the functional activity of methylenetetrahydrofolate reductase (MTHFR) in one-carbon metabolism. The availability of methyl groups for DNA methylation reactions is mainly regulated by the activity of MTHFR, which is indeed a fundamental enzyme in one-carbon metabolism. Its substrate, 5,10-methylenetrahydrofolate, is necessary for thymidylate and purine synthesis and its enzymatic product regulates the folate-dependent *de novo* synthesis of 5-methyltetrahydrofolate, the prominent circulatory form of folate and the precursor of *S*-adenosylmethionine, universal methyl donor for several biological methylation reactions, including that of DNA (8). We recently described that the different availability of methylated forms of folate in homozygous mutant carriers of the *MTHFR 677C>T* polymorphism (9) affects genomic DNA methylation through gene-nutrient interaction (10, 11). We observed that carriers of the *677TT* genotype have a diminished level of DNA methylation compared with those with the *677CC* wild-type genotype but only the *677TT* subjects with low levels of folate accounted for the diminished DNA methylation, thus indicating that the *MTHFR 677C>T* polymorphism influences DNA methylation level through an interaction with folate status (11, 12).

A second common mutation in the *MTHFR* gene, *1298A>C*, was reported. First described in association with ovarian carcinoma (13), it was subsequently investigated for its association with risk of neural tube defects, a group of diseases

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closely linked to one-carbon metabolism functions (14). However, whether or not these associations are ultimately due to a reduced activity of the mutant *MTHFR 1298A>C* enzyme is yet controversial (14-18). Most recently, polymorphic sites in folate metabolizing genes, particularly those affecting MTHFR activity, have been associated with a reduced risk of adult (19-22) and childhood (23-25) acute lymphoblastic leukemia development as well as with modulation of the response to chemotherapy (26). Furthermore, epidemiologic studies have shown an association between *MTHFR* polymorphisms and susceptibility to cancer affecting also other tissues (27-30). Because of the fundamental role of folate in nucleic acid synthesis and repair systems (31) as well as DNA methylation (12), alterations in this metabolic pathway seem an elective candidate target for the understanding of folate-related carcinogenesis with potential diagnostic and therapeutic implications (25, 26). Furthermore, it is known that *MTHFR 677C>T* and folate, independently or interactively, are important determinants of genome stability (7), cell proliferation, and chromosomal breakage, particularly in lymphoid cells (32-35).

Recent reports indicate that the *MTHFR 1298A>C* polymorphism is associated with a decreased risk of acute lymphoblastic leukemia in adults and children carrying the mutant allele either in heterozygosity (*1298AC*) or homozygosity (*1298CC*; refs. 19, 24). Furthermore, it has been reported that individuals with the combined *1298AA/677TT* or *1298CC/677CC* genotype show respectively a 2.5- and 3.3-fold reduction of risk for childhood acute lymphoblastic leukemia (25). Although several hypotheses have been proposed, there is yet no clear evidence for a link between this polymorphism and DNA methylation status.

We designed the present study precisely with the aim of evaluating the interaction between the *MTHFR 1298A>C* mutation and folate in lymphocyte DNA methylation status as a potential mechanism by which this polymorphism exerts a protective effect, particularly on the risk of lymphoid cell malignancies, either alone or in combination with the *MTHFR 677C>T*.

Materials and Methods

Study Population. We studied 198 unrelated, age- and sex-matched subjects from a single geographic area and recruited those referred to the Section of Internal Medicine of the Clinical and Experimental Medicine Department and to the Institute of Cardiology and Cardiovascular Surgery of the University of Verona (Verona, Italy), as previously described (36). Subjects for whom a complete set of biochemical data pertaining to folate-homocysteine metabolism (plasma folate levels, vitamin B12, and total plasma homocysteine concentrations) and a complete clinical history were available, were selected for the present study on gene-nutrient interaction between folate and *MTHFR* polymorphisms. Subjects were considered as a whole with no distinction referred to their pathologic status, and excluded from the study were those with conditions known to influence homocysteine levels such as those subjects taking folate or vitamin B_{12} or any multivitamin supplementation, those with any major acute illness, and those using drugs interfering with folate metabolism (i.e., penicillamine, anticonvulsants, and methotrexate). Informed consent was obtained from all the subjects and the study received the approval of our Institutional Review Board.

Biochemical Analyses. Samples of venous blood were drawn from each subject after an overnight fast. DNA was extracted from peripheral lymphocytes using a phenol/chloroform/isoamyl alcohol protocol. All the laboratory methods for biochemical analyses have been done as previously described (36). Levels of total homocysteine (tHcy) were measured by high-performance liquid chromatography with fluorescent detection according to Araki and Sako (37). Plasma folate was measured by an automated chemiluminescence method (Chiron Diagnostics, East Walpole, MA).

Methylenetetrahydrofolate Reductase Genotyping. For the *MTHFR 677C>T* single nucleotide polymorphism, we used primer sets, PCR-RFLP method, and nomenclature as previously described by Frosst et al. (38), after optimizing the conditions in our laboratory. The *1298A>C* mutation analysis was done as described by Weisberg et al. (ref. 15; i.e., by PCR followed by *Fnu*4HI endonuclease restriction). Positive and negative mutation controls were always included in each gel during the study for the genotyping of both the *677C>T* and *1298A>C* mutations (15).

DNA Methylation Measurement. To measure genomic DNA methylation, we used a recently developed liquid chromatography electrospray ionization mass spectrometry method that allows to define DNA methylation status as the absolute amount (in nanograms) of 5-methyl-2′-deoxycytidine (mCyt) per microgram of DNA (11, 39). Briefly, 1 μg of DNA was hydrolyzed by sequential digestion with three nucleases. DNA hydrolyzates were then injected onto an analytic column and subsequently separated by reversed-phase high-performance liquid chromatography in isocratic mode. The four major DNA bases and 5-methyl-2′-deoxycytidine were resolved and eluted in a short time run. The identification of 5-methyl-2′-deoxycytidine was obtained by spectral analysis of chromatographic peaks by isotope-labeled compounds as internal standard (39).

Statistical Analysis. All computations were done by using the Systat software 10.0 program for Windows (2000, SPSS, Inc., Chicago, IL). The distribution of continuous variables in groups is expressed as mean ± SD. Analysis was done with log-transformed data for all skewed variables including DNA methylation. Geometric means (antilogarithms of the transformed means) are presented, and 95% confidence intervals are calculated using the transformed values and displayed as the antilogarithms of the transformed data. Statistical significance for differences in continuous variables was tested by Student's unpaired t test or by AN(C)OVA with Tukey's post hoc comparison of the means when appropriate. Categorical variables were analyzed using a χ^2 test or linear regression analysis when appropriate. Statistical significance refers to a two-tailed analysis where $P < 0.05$.

Results

Each end point was evaluated for the three *MTHFR 1298A>C* polymorphism genotypes (*1298AA*, *1298AC*, and *1298CC*; Table 1; Fig. 1) as well as for the three different combined genotype groups for *1298A>C* and *677C>T* polymorphisms (*1298AA/677TT*, *1298AA/677CC*, and *1298CC/677CC*; Table 2; Fig. 2).

Frequency of Each *MTHFR* Polymorphism and Combined *MTHFR* Genotype Distribution

MTHFR 1298A>C Polymorphism. The overall allele frequency of the *1298C* allele in the studied population was 0.33 and the genotype frequency was as follows: *1298AA*, 46% (n = 91); *1298AC*, 33% (n = 65); and *1298CC*, 21% (n = 42).

Combination of MTHFR 1298A>C and 677C>T Polymorphisms. Among 91 *1298AA* subjects, 19 (21%) carried a *677CC* genotype and 72 (79%) carried a *677TT* genotype. The *677T* and *1298C* alleles only occurred in *trans*; no individual presented a *1298CC/677TT* genotype configuration.

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Table 1. Biochemical features of the study subjects according to *MTHFR 1298A>C* genotypes

	1298AA, N = 91	*1298AC*, N = 65	*1298CC*, N = 42	P (*AA* vs *AC*)	P (*AA* vs *CC*)	P (*AC* vs *CC*)
Plasma folate (nmol/L)	10.90 (9.99-11.89)	12.19 (11.03-13.44)	12.40 (10.92-14.08)	0.094	0.097	0.828
Vitamin B_{12} (pmol/L)	448 (404-495)	378 (346-413)	428 (380-482)	0.013	0.591	0.097
Vitamin B_6 (nmol//L)	28.16 (24.87-31.88)	28.47 (25.00-32.42)	29.10 (23.49-36.08)	0.900	0.772	0.840
tHcy (μmol/L)	18.71 (16.77-20.86)	13.97 (12.95-15.08)	14.45 (12.91-16.19)	<0.0001	0.001	0.603
Genomic DNA methylation (ng mCyt/μg DNA)	3.72 (2.73-5.07)	8.59 (6.83-10.77)	6.79 (4.96-9.28)	<0.0001	0.007	0.226

NOTE: Plasma folate, vitamin B12, vitamin B6, tHcy, and genomic DNA methylation are presented as geometric means (antilogarithms of the transformed means) and 95% confidence intervals are reported in parentheses with two-tailed P values. Statistical difference was evaluated by Student's t test except when differently indicated. Statistical difference was evaluated by χ^2 test.

Plasma Folate Concentrations

MTHFR 1298A>C Polymorphism. As shown in Table 1, plasma folate concentrations did not differ among the three genotype groups. However, when the subjects carrying the *1298C* allele either in hetero- or homozygosity were considered together, plasma folate concentration within the *1298AA* group was significantly lower than that found in those carrying at least one *1298C* allele (10.87 versus 12.22 nmol/L, P < 0.05).

Combined MTHFR 1298A>C and 677C>T Polymorphisms. Plasma folate concentration was lower in the *1298AA/677TT* group compared with the *1298CC/677CC* group (10.73 versus 12.62 nmol/L, P = 0.043; Table 2). Comparisons of *1298AA/677TT* with *1298AA/677CC* and *1298CC/677CC* groups did not show any significant difference (Table 2).

Total Plasma Homocysteine Concentrations

MTHFR 1298A>C Polymorphism. As shown in Table 1, the concentration of tHcy in the *1298AA* group was significantly higher than that in either *1298AC* or *1298CC* group (18.71 versus 13.97 μmol/L and 18.71 versus 14.45 μmol/L, P < 0.0001 and P = 0.001, respectively). The difference was significant even when the carriers of the *1298C* allele were considered together (*1298AA* versus *1298AC*/*1298CC*; 18.71 versus 14.16 μmol/L, P < 0.0001). When folate levels were taken into consideration, the *1298AA* subjects with low folate concentrations (i.e., below the median value, 12 nmol/L) showed a significantly higher tHcy concentration compared with that of *1298AA* individuals with adequate folate levels (i.e., above the median value, 12 nmol/L; 22.08 versus 13.18 μmol/L, P < 0.0001). Within the *1298CC* genotype carrier group, those with low plasma folate levels also had higher tHcy concentrations compared with those with high folate concentrations (19.46 versus 13.93 μmol/L, P < 0.0001). However, there was a significant difference in tHcy concentrations between *1298AA* and *1298CC* genotypes (19.29 versus 15.89 μmol/L, P = 0.002) at the low level of folate, but no significant difference between *1298AA* and *1298AC* (19.29 versus 16.27, P = 0.11). Such difference was no longer present if the comparison was made between subjects with levels of folate above the median (13.63 versus 12.62 μmol/L, P = 0.321, and 13.63 versus 13.53 μmol/L, P = 0.891, for the comparison between *1298AA* versus *1298CC* and *1298AA* versus *1298AC*, respectively).

Combination of MTHFR 1298A>C and 677C>T Polymorphisms. Total plasma homocysteine concentration in the *1298AA/677TT* group was significantly higher than that in the *1298AA/677CC* and *1298CC/677CC* group (19.57 versus 15.79 μmol/L, P = 0.045, and 19.57 versus 14.41 μmol/L, P = 0.001, respectively; Table 2). Even when the *1298AA/677TT* was compared with the *1298AA/677CC* and *1298CC/677CC* groups taken together, the difference remained statistically significant (19.57 versus 14.86 μmol/L, P = 0.001). However, no difference was detected for the comparison of the *1298AA/677CC* with the *1298CC/677CC* group (15.79 versus 14.41 μmol/L, P = 0.358; Table 2). When we analyzed the data according to plasma folate concentrations, the *1298AA/677TT* group showed a significant difference between low- and high-folate groups (23.87 versus 13.09 μmol/L, P < 0.0001). A difference according to low or high plasma folate levels was also detected within the *1298CC/677CC* group (16.87 versus 12.55 μmol/L, P = 0.012). However, no differences were found within the *1298AA/677CC* group (16.03 versus 13.49 μmol/L, P = 0.257).

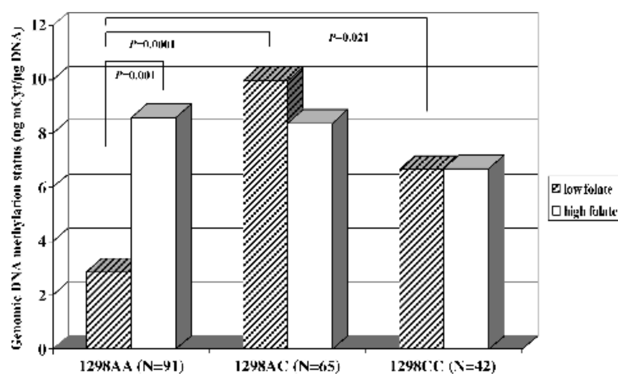

Figure 1. Genomic DNA methylation according to *MTHFR 1298A>C* genotype and low or high plasma folate levels (i.e., below or above the median, 12 nmol/L). Statistical difference was evaluated by Student's t test. Within the *1298AA* group, the levels of DNA methylation were significantly reduced in the low-folate group compared with the high-folate group (2.87 versus 8.59 ng mCyt/μg DNA, P = 0.001). No differences were detected within both the *1298AC* and *1298CC* genotypes according to folate levels (P = 0.771 and P = 0.135, respectively). Considering only the three genotype groups at low folate status, the levels of DNA methylation were significantly lower in *1298AA* versus *1298AC* and *1298AA* versus *1298CC* (P = 0.0001 and P = 0.021, respectively). Among high-folate groups, there were no statistically significant differences for *1298AA* versus *1298AC* (P = 0.98) and *1298AA* versus *1298CC* (P = 0.084).

Genomic DNA Methylation

MTHFR 1298A>C Polymorphism. DNA methylation status within *MTHFR 1298AA* group was significantly decreased compared with that of either *1298AC* (3.72 versus 8.59 ng mCyt/μg DNA, P < 0.0001) or *1298CC* (3.72 versus 6.79 ng mCyt/μg DNA, P = 0.007; Table 1). DNA methylation was significantly decreased in the *MTHFR 1298AA* group compared with the *1298AC* and *1298CC* genotypes considered together (3.72 versus 7.82 ng mCyt/μg DNA, P < 0.0001). There was no significant difference between the *1298AC* and *1298CC* groups for DNA methylation status. However, when plasma folate levels were taken into account, the reduced DNA methylation in the *1298AA* group was evident only for those subjects with low plasma folate concentrations (below the

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Table 2. Biochemical data according to the combined presence of *MTHFR 1298A>C* and *677C>T* genotypes

	Group 1 (*1298AA/677TT*), N = 72	Group 2 (*1298AA/677CC*), N = 19	Group 3 (*1298CC/677CC*), N = 42	P (group 1 vs group 2)	P (group 1 vs group 3)	P (group 2 vs group 3)
Plasma folate (nmol/L)	10.73 (9.73-11.85)	11.52 (9.31-14.25)	12.62 (11.15-14.30)	0.513	0.043	0.440
Vitamin B_{12} (pmol/L)	437 (391-487)	487 (369-642)	433 (383-488)	0.450	0.900	0.419
Vitamin B_6 (nmol//L)	30.53 (26.62-35.02)	21.03 (16.05-27.52)	28.59 (23.01-35.51)	0.016	0.607	0.090
tHcy (μmol/L)	19.57 (17.18-22.28)	15.79 (13.34-18.70)	14.41 (12.83-16.18)	0.045	0.001	0.358
Genomic DNA methylation (ng mCyt/μg DNA)	3.11 (2.16-4.49)	7.29 (4.64-11.44)	7.14 (5.26-9.67)	0.004	0.001	0.936

NOTE: Plasma folate, vitamin B12, vitamin B6, tHcy, and genomic DNA methylation are presented as geometric means (antilogarithms of the transformed means) and 95% confidence intervals are reported in parentheses with two-tailed P values. Statistical difference was evaluated by Student's t test except when differently indicated. Statistical difference was evaluated by χ^2 test.

median value, i.e., 12 nmol/L), whereas under conditions of adequate plasma folate, the levels of genomic DNA methylation were similar to that of the other two *MTHFR* genotypes (Fig. 1).

To further evaluate the relationship between folate and *MTHFR* genotypes, we did additional analyses. After dividing the population sample into tertiles of plasma folate according to the three different *1298A>C* genotypes, we observed that only among the individuals carrying the *1298AA* genotype was there a graded effect of folate in determining levels of DNA methylation (2.39 ng mCyt/μg DNA for the lowest tertile, 5.33 ng mCyt/μg for the intermediate tertile, and 7.54 ng mCyt/μg DNA for the highest tertile, P for trend = 0.005). Among the carriers of the *1298AC* or *1298CC* genotype, however, DNA methylation status was not different across the increasing tertiles of plasma folate.

Combination of MTHFR 1298A>C and 677C>T Polymorphisms. Genomic DNA methylation status of the *MTHFR 1298AA/677TT* group was significantly decreased compared with that of *1298AA/677CC* (3.11 versus 7.29 ng mCyt/μg DNA, P = 0.001) and *1298CC/677CC* groups (3.11 versus 7.14 ng mCyt/μg DNA, P = 0.0001; Table 2). There was no significant difference between the *1298AA/677CC* and *1298CC/677CC* groups (Table 2). However, when considering plasma folate levels, the reduced DNA methylation was evident only for the *1298AA/677TT* subjects with low plasma folate concentrations (*1298AA/677TT*, 2.44; *1298AA/677CC*, 5.57; and *1298CC/677CC*, 7.53 ng mCyt/μg DNA), whereas under conditions of adequate plasma folate, genomic DNA methylation status was similar to the other two *MTHFR* genotype groups (*1298AA/677TT*, 8.31 ng mCyt/μg DNA; *1298AA/677CC*, 9.49 ng mCyt/μg DNA; and *1298CC/677CC*, 6.69 ng mCyt/μg DNA; Fig. 2). No statistically significant difference for DNA methylation status was observed within the *1298AA/677CC* and *1298CC/677CC* groups between low and high levels of folate (5.57 versus 9.49 ng mCyt/;g DNA and 7.53 versus 6.69 ng mCyt/μg DNA, P = 0.67 and P = 0.69, respectively; Fig. 2).

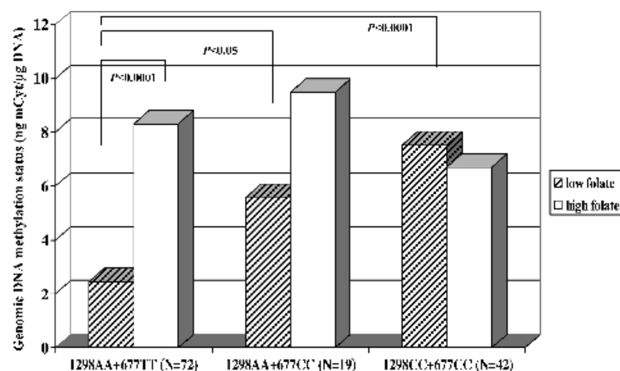

Figure 2. Genomic DNA methylation (ng mCyt/μg DNA) according to combined *MTHFR 1298A>C* and *677C>T* genotypes and low or high plasma folate levels (i.e., below or above the median, 12 nmol/L). Statistical difference was evaluated by Student's t test. The reduced DNA methylation was evident only for *1298AA/677TT* genotype carriers with low plasma folate, whereas among *1298AA/677TT* subjects under adequate conditions of plasma folate, the levels of genomic DNA methylation were similar to that of the other two *MTHFR* genotype groups. The difference in DNA methylation between low- and high-folate subjects in the *1298AA/677TT* group was statistically significant (2.44 versus 8.31 ng mCyt/μg DNA, P < 0.0001).

Discussion

Epidemiologic studies recently observed an association of polymorphic variants of the *MTHFR* gene with reduced risk of adult and childhood acute lymphoblastic leukemia (19, 22-25) and with the modulation of risk of other forms of cancer (27-30, 40, 41). The *MTHFR 677TT* homozygote mutant genotype has been consistently reported to have a protective effect against the risk of cancer of the colorectum (27, 42) and of the lymphoid tissue in several studies (19, 20, 22-25). The role of *MTHFR* polymorphisms in modulating cancer risk, however, is strongly associated with folate status, so that the protective effect of the *677TT* genotype, under adequate folate conditions, turns to a situation of elevated risk of cancer of the colorectum among *MTHFR 677TT* carriers with low folate status or intake (27, 43). On the other hand, although some studies observed a protective effect also for the *MTHFR 1298CC* mutant genotype, results in this regard are conflicting (22, 23). Because the function of MTHFR is essential for providing methyl groups for DNA methylation (27), it is highly plausible that enzymatic variants due to functional polymorphisms may alter this major epigenetic feature of DNA, patterns of which greatly affect carcinogenesis (1, 3, 5). Different from *677C>T*, the biochemical effect of the *1298A>C* mutation on MTHFR function is still controversial (14-18), and its role in the risk of cancer is therefore more questionable (40, 41).

In the present study, we sought to evaluate the *MTHFR 1298A>C* polymorphism interaction with folate status and the subsequent effects on genomic methylation in human lymphocyte DNA as a potential mechanism underlying the association between the *MTHFR 1298A>C* polymorphism and the risk of cancer. We observed that lymphocyte DNA from subjects with the *MTHFR 1298AA* wild-type genotype has reduced genomic DNA methylation compared with that of carriers of either the *1298AC* or *1298CC* genotype. This finding referred only to those subjects whose plasma folate concentrations are low with no difference in DNA methylation among the three *1298A>C* genotypes when stratified for high levels of folate. A further evidence of such gene-nutrient interaction was the detection of a graded effect of

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folate in determining DNA methylation, as shown by the increasing DNA methylation levels across increasing tertiles of plasma folate concentrations. This gene-nutrient interaction phenomenon affecting DNA methylation is supported also by the observation of a similar effect of the *1298AA* genotype on tHcy levels, as shown by the higher tHcy in the *1298AA* group compared with carriers of at least one *1298C* allele, as similarly described for the *677TT* genotypes (36). Because tHcy is a product of the intracellular hydrolysis of *S*-adenosylhomocysteine, which is known to be an inhibitor of DNA methylation, tHcy levels could be also considered as a marker of tissue DNA methylation status, as reported by Yi et al. (44).

The most crucial conflict in the present observation, however, is that the *MTHFR 1298AA* wild-type genotype for the *MTHFR 1298A>C* mutation shows a model of interaction with folate similar to that of the mutant *MTHFR 677TT* genotype. When considered by combined polymorphic genotypes, the *1298AA/677TT* group showed decreased plasma folate concentration, increased tHcy, and decreased DNA methylation status compared with the *1298AA/677CC* and *1298CC/677CC* groups, and even when folate concentration was taken into account, only the *1298AA/677TT* genotype group showed a decreased genomic DNA methylation when plasma folate levels are low. Furthermore, when we compared the *1298AA/677CC* and *1298CC/677CC* groups, to avoid any possible influence of the mutant *677TT* genotype, there was no difference in plasma folate, tHcy, and lymphocyte genomic DNA methylation. These data suggest that the *MTHFR 1298A>C* polymorphism does not impair the enzyme function significantly enough to affect the pathway of homocysteine remethylation and biological methylation functions, thus substantiating previous findings showing no effect of the *1298A>C* polymorphism *per se* in determining tHcy (14, 18), and leading to the understanding of a minor effect of the *1298A>C* mutation on this pathway of one-carbon metabolism as a consequence of reduced MTHFR enzyme function.

Plausible explanations for the similar features observed for the *1298AA* and *677TT* arise from the analysis of the distribution of the *677C>T* polymorphism among carriers of the *1298A>C* polymorphism. Noteworthy, we observed that among *1298AA* carriers, 79% had a *677TT* genotype, and all of the *1298AC* and *1298CC* carriers had a *677CC* genotype. Furthermore, the *1298C* and *677T* alleles only occurred in *trans* in our population, consistent with several previous reports on the very rare occurrence of the two polymorphisms on the same allele (14, 15, 18). This combined genotype setting, therefore, may explain the similar pattern of DNA methylation for the *1298AA* and *677TT* genotypes, and that of the *1298CC* with the coexistence of the *677CC* genotype. Indeed, such observations confirm as well the population specificity of these genotypes and could give reason for the controversial association and potentially ethnic-related association of *MTHFR* genotypes with acute lymphoblastic leukemia in some reports (22, 45).

Consistent with the results of the present study were the reports on the structural and biochemical characteristics of the two different enzyme variants, *MTHFR 677C>T* and *1298A>C* (46, 47). The *677C>T* polymorphism lies at the base of binding site for the MTHFR cofactor, flavin adenine dinucleotide, and has been shown to affect the MTHFR enzyme activity more significantly than the *1298A>C* variant (14, 46). Individuals carrying the *677TT* genotype show a 30% *in vitro* enzyme activity compared with the *677CC* wild-type (38, 46). The *MTHFR 1298A>C* variant, instead, has been shown to have biochemical properties that are not markedly distinguishable from those of the *1298AA* wild-type genotype (47). Further reports on the biochemical relevance of the *1298A>C* mutation showed also that lymphocytes from individuals carrying the mutant *1298CC* genotype have ~60% specific wild-type MTHFR activity *in vitro* compared with the *1298AA* wild-type (14), although such a decrease did not seem sufficient to affect tHcy levels (14, 15, 18). Thus, considering the strong effect of the *MTHFR 677C>T* variant on determining the availability of methylated forms of folate and on remethylation pathway (9, 11), we can speculate that the genomic DNA methylation pattern in *1298AA* genotypes is mostly driven by the concomitant presence of the *677TT* genotype. The effect of the *1298AA/677TT* genotype also is likely due primarily to the high portion of *677TT* in the combined genotype group, and the effect of both *1298AA/677CC* and *1298CC/677CC* combined genotypes is mainly related to the simultaneous presence of the *677CC* genotype.

Therefore, it may be of crucial importance for future epidemiologic studies to evaluate both *MTHFR* single polymorphisms as well as combined genotype configurations in the *MTHFR* gene (48) and to simultaneously consider environmental factors such as folate status (49). Indeed, whereas a strong gene-nutrient interaction affects the *MTHFR 677C>T* polymorphism in determining DNA methylation, the mildly decreased activity due to *MTHFR 1298A>C* mutation may affect a different pathway in one-carbon metabolism, such as that of DNA synthesis, and therefore accounts for a different mechanism for the reduced risk of leukemia in carriers of the *1298C* allele. Specific mechanistic studies are needed to clarify the molecular bases of such associations.

The evaluation of *MTHFR* common mutations and folate status should be considered in the view of an individually tailored assessment of cancer risk because it may as well lead to the ultimate goal of providing, in the near future, a better clinical and preventive approach (50).

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