Common Gene Polymorphisms in the Metabolic Folate and Methylation Pathway and the Risk of Acute Lymphoblastic Leukemia and non-Hodgkin's Lymphoma in Adults

Donato Gemmati,[1] Alessia Ongaro,[2] Gian L. Scapoli,[1] Matteo Della Porta,[1] Silvia Tognazzo,[1] Maria L. Serino,[1] Eros Di Bona,[3] Francesco Rodeghiero,[3] Giuseppe Gilli,[4] Roberto Reverberi,[5] Angelo Caruso,[2] Michela Pasello,[2] Agnese Pellati,[2] and Monica De Mattei[2]

[1]Department of Biomedical Sciences and Advanced Therapies, Center Study for Hemostasis and Thrombosis and Unit of Hematology, and [2]Department of Morphology and Embryology, Unit of Histology, University of Ferrara, Ferrara, Italy; [3]Department of Hematology, San Bortolo Hospital, Vicenza, Italy; and [4]Department of Health Physics and [5]Transfusion Center, Sant'Anna Hospital, Ferrara, Italy

Abstract

Folate and methionine metabolism is involved in DNA synthesis and methylation processes. Polymorphisms in the genes of folate metabolism enzymes have been associated with some forms of cancer. In a case-control study, we evaluated whether four common polymorphisms in methylenetetrahydrofolate reductase (*MTHFR* C677T and A1298C), methionine synthase (*MS* A2756G), and methionine synthase reductase (*MTRR* A66G) genes may have a role in altering susceptibility to adult acute lymphoblastic leukemia (ALL) and non-Hodgkin's lymphoma (NHL). We analyzed DNA of 120 adult ALL, 200 NHL, and 257 healthy control subjects. Individual carrying the *MTHFR* 677TT genotype showed a 3.6-fold decreased ALL risk [odds ratio (OR) 0.28, 95% confidence interval (95% CI) 0.12–0.72] than wild-types. Similarly, *MS* 2756GG individuals showed a 5.0-fold decreased ALL risk (OR 0.20, 95% CI 0.02–1.45) than wild-types. In combined results, subjects with the *MTHFR* 677CT/TT and *MS* 2756AG/GG genotypes revealed a 3.6-fold ALL risk reduction (OR 0.28, 95% CI 0.14–0.58) and those with the *MTHFR* 677TT and *MTRR* 66AG genotypes revealed a 4.2-fold ALL risk reduction (OR 0.24, 95% CI 0.06–0.81). Finally, those with the *MS* 2756AG/GG and *MTRR* 66AG/GG genotypes revealed a 2.2-fold ALL risk reduction (OR 0.45, 95% CI 0.10–0.85). Single analysis for NHL did not show any significant difference for all the polymorphisms investigated, but in the low-grade NHL subgroup, we found a 2.0-fold risk reduction for the *MTRR* 66GG homozygous genotype (OR 0.50, 95% CI 0.25–0.99), which was higher (OR 0.37, 95% CI 0.14–0.85) when analyzed in combination with *MS* 2756AA genotype. These data are in accordance with the hypothesis that polymorphisms in the genes for folate and methionine metabolism might play a greater role in the occurrence of ALL than NHL by influencing DNA synthesis and/or DNA methylation. (Cancer Epidemiol Biomarkers Prev 2004;13(5):787–94)

Introduction

The biological mechanisms and etiology of malignant lymphoma and leukemia are not yet completely understood and ionizing radiation, benzene, and cancer chemotherapy account for only a small percentage of the total cases (1, 2). Single genetic defects are unlikely to be responsible for hematological malignancies and a modest familial link is observed. Therefore, unfavorable gene-environment interactions might be involved in the genesis of these tumors. Several genetic alterations, due to incorrect DNA synthesis or altered methylation status of oncogenes and/or tumor suppressor genes, have been identified in the pathogenesis of lymphoid malignancies (3–5). Folate and methionine metabolism plays an essential role in both DNA synthesis and methylation processes. Defects or polymorphisms in the genes of the folate-dependent enzymes and deficiencies of micronutrients may influence cancer susceptibility (5–8). Methylenetetrahydrofolate reductase (*MTHFR*) reduces 5,10-methylenetetrahydrofolate (methylene-THF) to 5-methyl-THF, the main circulatory form of folate and one-carbon donor for the remethylation of homocysteine to methionine (Fig. 1). Two common polymorphisms (C677T and A1298C) in the *MTHFR* gene (9, 10) have been described which reduce enzyme activity, the latter to a lesser extent than C677T. A lower *MTHFR* activity leads to increased plasma levels of homocysteine and decreased 5-methyl-THF formation. This contributes to the pool of methylene-THF, the methyl group donor

Received 8/30/03; revised 1/12/04; accepted 1/15/04.

Grant support: Italian Ministry for Universities and Scientific and Technological Research funds and Fondazione Cassa di Risparmio di Ferrara grant.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Presented in part at the Fourth International Conference on Homocysteine Metabolism, Basel, Switzerland, June 29–July 3, 2003.

Requests for reprints: Donato Gemmati, Department of Biomedical Sciences and Advanced Therapies, Center Study for Hemostasis and Thrombosis, University of Ferrara, C.so Giovecca 203, I-44100 Ferrara, Italy. Phone: 39-0532-237-291; Fax: 39-0532-209-010. E-mail: d.gemmati@unife.it

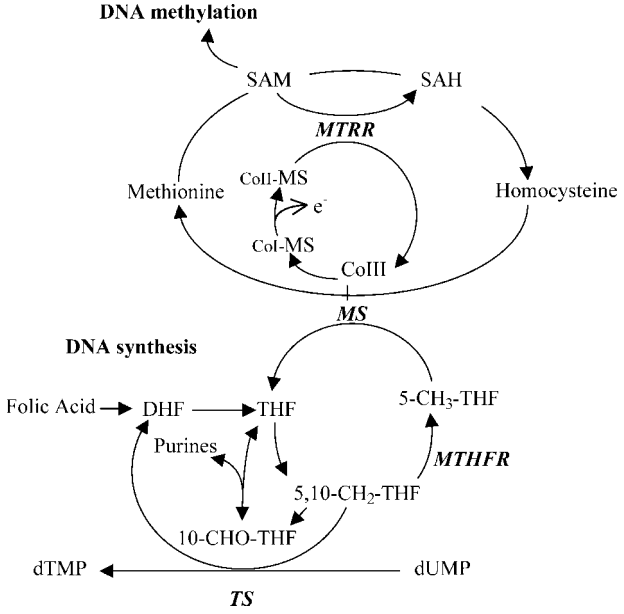

Figure 1. Overview of folate and methionine metabolism pathways. *TS,* thymidylate synthase; *DHF,* dihydrofolate; *THF,* tetrahydrofolate; *5,10-CH_2-THF,* 5,10-methylenetetrahydrofolate; *5-CH_3-THF,* 5-methyltetrahydrofolate; *10-CHO-THF,* 10-formyltetrahydrofolate; *CoI,* cob(I)alamin; *CoII,* cob(II)alamin; *CoIII,* cob(III)alamin; *SAM, S*-adenosylmethionine; *SAH, S*-adenosylhomocysteine.

for the conversion of dUMP to dTMP by thymidylate synthase enzyme. Therefore, it is thought that lower *MTHFR* activity might favor optimal DNA synthesis by reducing uracil misincorporation rate, a potential cause of double-strand breaks during uracil excision repairing processes (6, 11). On the other hand, a lower availability in 5-methyl-THF might decrease the synthesis of methionine and consequently of *S*-adenosylmethionine, crucially involved in cellular methylation processes (12, 13). Methionine synthase (*MS*) catalyzes the transfer of the methyl group from 5-methyl-THF to homocysteine generating tetrahydrofolate and methionine, thus playing a critical role in maintaining adequate intracellular methionine concentrations. A recently identified common polymorphism (*MS* A2756G) is thought to affect the enzymatic activity (14) and to induce modest homocysteine reduction (15) and DNA hypomethylation (16). While several studies have investigated the association between folate-related polymorphisms and neural tube defect (17–19), arterial or venous vascular disease (9, 20–24), and several forms of solid tumor (25–28), only few recent studies have investigated their influence on the risk of lymphoid malignancies. Skibola *et al.* (29) and Matsuo *et al.* (16) reported independently that individuals with either *MTHFR* polymorphisms had a significant lower susceptibility to adult acute lymphoblastic leukemia (ALL) and malignant lymphoma, respectively. Similar results were obtained by Franco *et al.* (30) and Wiemels *et al.* (31) in childhood leukemia. The role of *MS* A2756G polymorphism appears more controversial. In fact, Matsuo *et al.* (16) have shown that *MS* A2756G polymorphism may be considered a risk factor for malignant lymphoma, while Skibola *et al.* (32) found a protective effect of *MS* A2756G against ALL when analyzed with serine hydroxymethyltransferase C1420T polymorphism. Finally, methionine synthase reductase (*MTRR*) might be a further candidate gene in studies on lymphoid malignancies susceptibility, alone or in conjunction with *MS* gene, because it maintains adequate cell levels of methylcob(III)alamin, the functional cofactorial form of *MS* enzyme (33, 34). Alterations in *MTRR* activity may affect *MS*-dependent remethylation processes (33, 35, 36); therefore, we also tested whether a newly discovered *MTRR* A66G polymorphism (37) might be a further modulating factor in hematological malignancies. In the present study, we analyzed the association of two folate-related polymorphisms (*MTHFR* C677T and A1298C) and two methylation-related polymorphisms (*MS* A2756G and *MTRR* A66G) with susceptibility to ALL and non-Hodgkin's lymphoma (NHL).

Methods

Selection of Cases and Controls. Recruitment of ALL cases consisted of 120 Italian individuals (all Caucasians) with newly diagnosed ALL according to the French-American-British classification. They were from the files of patients who received hematological diagnosis by the Unit of Hematology of the University of Ferrara (n = 71) in the period between January 1990 and December 2001 and by the Department of Hematology of the San Bortolo Hospital of Vicenza (n = 49) in the period between December 1992 and December 2001. The two hematological centers are in adjacent regions of northern Italy about 100 km distant and all the cases were resident in the same geographic area. The whole group of ALL cases were persons aged 18–75 years with a mean age of 48.5 $\pm$ 15.64 years and 57.5% of them were male. Among the ALL cases, 88 (73.3%) were diagnosed as ALL-B and 20 (16.6%) were ALL-T, and for 12 cases (10%), the immunophenotype could not be specified.

Recruitment of NHL cases consisted of 200 Italian individuals (all Caucasians) with newly diagnosed NHL according to the Revised European American Lymphoma classification. They were from the files of patients who received hematological diagnosis by the Unit of Hematology of the University of Ferrara in the period between June 1987 and December 2001. NHL cases were persons aged 18–75 years with a mean age of 54.5 $\pm$ 15.32 years and 59% of them were male. Among the NHL cases (B-NHL n = 184 and T-NHL n = 16), 102 (51.0%) were classified as low-grade NHL (follicular lymphoma n = 53, marginal zone B-cell lymphoma n = 21, mantle cell lymphoma n = 16, lymphoplasmocytic lymphoma n = 11, Castleman lymphoma n = 1) and the remaining 98 (49.0%) cases were classified as high-grade NHL (diffuse large B-cell lymphoma n = 79, anaplastic large cell lymphoma n = 19). Cases diagnosed with ALL or NHL within 6 months of being diagnosed with a prior hematological malignancy or within 2 years of any other cancer were considered ineligible.

The control group (n = 257) consisted of Italian healthy individuals (all Caucasians) from the same geographic area as the cases, not relatives of the cases and randomly selected from the blood donor lists and in the same proportion as the patients coming from the two hospitals considered in the study in the period between January

1999 and December 2001. They were aged 18–75 years with a mean age of 51.5 ± 16.26 years and 58.0% of them were male. They did not have significant differences concerning sex and age distribution. Peripheral blood samples for all cases were collected at the date of diagnosis before any pharmacological treatment and for controls at the moment of blood donation by venipuncture. All cases and controls at the moment of the peripheral blood collection gave informed consent to participate to research studies. The overall participation rate among cases was 97.2% (96.7% and 97.6% for ALL and NHL cases, respectively). As biological samples are concerned, the overall collection rate for cases was 99.3% (98.3% and 100% for ALL and NHL cases, respectively). Finally, the joint participation rate for cases was 96.6%. Among the randomly selected 280 controls, 265 (94.6%) were eligible and 97.0% of them (n = 257) provided biological samples. Globally, our survey had a joint participation rate of 94.4%.

Genotype Analyses. The shortage of stored blood from some ALL samples did not allow obtaining of sufficient DNA quantity to carry out all the PCR amplifications and/or control regenotyping. Therefore, a few of the ALL samples were not PCR amplifiable for all four polymorphisms, particularly for the oldest ones (specified at the bottom of Table 1).

DNA was isolated by using proteinase K treatment followed by phenol/chloroform extraction and ethanol precipitation. The genotyping protocol for *MTHFR* C677T polymorphism detection was adapted from Frosst *et al.* (9) with the following primers: 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ and 5′-AGG ACG GTG CGG TGA GAG TG-3′. The PCR conditions were modified as follows: 5 min initial at 94°C followed by 30 cycles of 94°C for 45 s, 65°C for 60 s, and 72°C for 90 s, and the PCR product was digested by *Hin*fI (Roche Diagnostics GmbH, Mannheim, Germany). The genotyping protocol for *MTHFR* A1298C polymorphism detection was adapted from Weisberg *et al.* (10) with the following primers: 5′-GGG AGG AGC TGA CCA GTG CAG-3′ and 5′-GGG GTC AGG CCA GGG GCA G-3′. The PCR conditions were modified as follows: 5 min initial at 94°C followed by 30 cycles of 94°C for 30 s, 59°C for 13 s, and 72°C for 17 s, and the PCR product was digested by *Fnu*4HI (New England BioLabs, Beverly, MA). The genotyping protocol for *MS* A2756G polymorphism detection was adapted from Matsuo *et al.* (16) with the following primers: 5′-TGT TCC AGA CAG TTA GAT GAA AAT C-3′ and 5′-GAT CCA AAG CCT TTT ACA CTC CTC-3′. The PCR conditions were modified as follows: 5 min initial at 94°C followed by 35 cycles at 95°C for 60 s, 60°C for 90 s, and 72°C for 60 s, and the PCR product was digested by *Hae*III (Roche Diagnostics). The genotyping protocol for *MTRR* A66G polymorphism detection was adapted from Jaques *et al.* (38) with the following primers: 5′-CAG GCA AAG GCC ATC GCA GAA GAC AT-3′ and 5′-CAC CTT CCC AAC CAA AAT TCT TCA AAG-3′. The PCR conditions were modified as follows: 5 min initial at 94°C followed by 32 cycles at 94°C for 60 s, 58°C for 45 s, and 72°C for 60 s, and the PCR product was digested by *Afl*III (New England BioLabs). All PCR cycles were performed in a Peltier Thermal Cycler apparatus (PTC-200; M. J. Research, Inc., Watertown, MA) and were completed with a 5 min final extension step at 72°C. DNA digestions were performed according to suppliers' instructions. Confirmation of genotypes was carried out by regenotyping a random selection of samples (cases and controls) for each polymorphism investigated. There were no discrepancies between genotypes determined in duplicate.

Statistical Analysis. Statistical differences between case and control populations were tested using χ^2 and Student's tests. Where appropriate, Yates' correction or Fisher's exact test were applied. $P \leq 0.05$ was considered statistically significant. Odds ratios (OR) and 95% confidence intervals (95% CI) were used to estimate the risk of developing ALL or NHL. Adjusted ORs for single or combined comparisons were calculated with logistic regression models that controlled for sex, age, and other polymorphisms (*i.e.*, *MTHFR* C677T and A1298C, *MTHFR* C677T and *MS* A2756G mutual OR adjustments, and *MTHFR* 677/*MS* 2756 interaction test). For the test trend across genotypes, from the reference genotype to the homozygous variant, P values are reported at the bottom of Table 1. The expected frequency of control genotypes were checked by the Hardy-Weinberg equilibrium test. All analyses were performed by Systat V.5.0 (Systat Inc., Evanston, IL) and SPSS Statistical Package (SPSS Inc., Chicago, IL).

Results

Comparison of the allele frequency and genotype distribution between the whole ALL group and the control group showed statistically significant differences. After categorizing the 120 ALL cases according to ALL-B and ALL-T immunophenotype, no significant differences were observed between the subgroups (data not shown); therefore, the ALL cases were analyzed as single group. In contrast, after subsetting the 200 NHL cases according to high/low grade of malignancy stratification, the *MTRR* and *MS* polymorphisms gave significant differences in the low-grade subgroup compared with controls. Therefore, NHL cases were analyzed both as a whole and a subdivided group. To evaluate in younger patients possible associations between the polymorphisms and ALL or NHL, we also subsetted cases in three subgroups of age (<40, <50, and <60 years old), but the subsetted analysis did not furnished significant differences among the subgroups.

Genotyping and Adjusted Risk Evaluation by Single Analysis for *MTHFR*, *MS*, and *MTRR* Polymorphisms in ALL Cases. Table 1 shows the allele and genotype frequencies for ALL cases and controls and the adjusted OR values for each polymorphism. Genotype frequencies were in agreement with the Hardy-Weinberg expectations in all the control genotypes analyzed.

MTHFR C677T The frequency of the 677T polymorphic allele was 31.1% in ALL cases and 44.7% in controls (P = 0.001). Considering the *MTHFR* 677CC genotype as the reference, individuals with *MTHFR* 677TT genotype showed a 3.6-fold reduction in ALL risk (OR 0.28, 95% CI 0.12–0.72) and individuals with *MTHFR* 677CT genotype showed a 1.7-fold reduction in ALL risk (OR 0.60, 95% CI 0.34–1.04). The test for trend yielded a significant P value of <0.001.

Table 1. Number of patients and controls, sex- and age-adjusted OR and 95% CI, for *MTHFR*, *MS*, and *MTRR* polymorphisms

Genotype	Cases [*n* (%); ALL (*n* = 120)]	OR	95% CI	*P*	Cases [*n* (%); NHL (*n* = 200)]	OR	95% CI	*P*	Controls [*n* (%); *n* = 257]
MTHFR 677 (*n* = 114)*									
CC	52 (45.6)	1.00	Reference		60 (30.0)	1.00	Reference		78 (30.3)
CT	53 (46.5)	0.60	0.34–1.04	0.055	101 (50.5)	0.95	0.65–1.58	0.82	128 (49.8)
TT	9 (7.9)	0.28	0.12–0.72	0.008	39 (19.5)	0.84	0.59–1.72	0.59	51 (19.8)
CT + TT	62 (54.4)	0.56	0.35–0.90	0.022	140 (70.0)	0.92	0.66–1.50	0.72	179 (69.6)
C	157 (68.8)	0.57	0.41–0.79	0.001	221 (55.2)	0.93	0.74–1.29	0.64	284 (55.3)
T	71 (31.1)				179 (44.8)				230 (44.7)
MTHFR 1298 (*n* = 114)*									
AA	47 (41.2)	1.00	Reference		96 (48.0)	1.00	Reference		126 (49.0)
AC	55 (48.2)	1.00	0.59–1.7	0.97	90 (45.0)	1.20	0.74–1.57	0.40	110 (42.8)
CC	12 (10.5)	0.90	0.36–2.20	0.81	14 (7.0)	0.78	0.40–1.71	0.36	21 (8.2)
AC + CC	67 (58.8)	1.07	0.65–1.77	0.78	104 (52.0)	1.15	0.73–1.49	0.62	131 (60.0)
A	149 (65.3)	1.27	0.89–1.73	0.16	282 (70.5)	0.98	0.71–1.36	0.93	362 (70.4)
C	79 (34.6)				118 (29.5)				152 (29.6)
MS 2756 (*n* = 118)[c]									
AA	88 (74.6)	1.00	Reference		129 (64.5)	1.00	Reference		158 (61.5)
AG	29 (24.6)	0.56	0.32–0.90	0.03	65 (32.5)	0.98	0.62–1.35	0.93	89 (34.6)
GG	1 (0.8)	0.20	0.02–1.45	0.14	6 (3.0)	0.78	0.23–2.11	0.67	10 (3.9)
AG + GG	30 (25.4)	0.52	0.30–0.80	0.011	71 (35.5)	0.96	0.58–1.27	0.85	99 (38.5)
A	205 (86.8)	0.55	0.37–0.87	0.008	323 (80.7)	0.94	0.61–1.22	0.76	405 (78.8)
G	31 (13.1)				77 (19.2)				109 (21.2)
MTRR 66 (*n* = 109)[b]									
AA	28 (25.7)	1.00	Reference		51 (25.5)	1.00	Reference		59 (22.9)
AG	58 (53.2)	1.13	0.63–2.02	0.68	106 (53.0)	1.13	0.65–1.61	0.64	122 (47.5)
GG	23 (21.1)	0.71	0.35–1.40	0.32	43 (21.5)	0.70	0.35–1.12	0.31	76 (29.6)
AG + GG	81 (74.3)	0.87	0.51–1.46	0.60	149 (74.5)	0.90	0.54–1.35	0.91	198 (77.0)
A	114 (52.3)	0.80	0.57–1.09	0.17	208 (52.0)	0.84	0.60–1.06	0.26	240 (46.7)
G	104 (47.7)				192 (48.0)				274 (53.3)

Note: OR for *MTHFR* 677 polymorphism has been adjusted for *MTHFR* 1298. In addition, *MTHFR* 677 polymorphism has been also adjusted for *MS* 2756 and vice versa. The trend test yielded a significant value ($P < 0.001$) only for *MTHFR* 677 and *MS* 2756 variants in ALL cases.
*Six patients were excluded from the analysis because DNA was not amplifiable by PCR (see Methods).
[c]Two patients were excluded from the analysis because DNA was not amplifiable by PCR (see Methods).
[b]Eleven patients were excluded from the analysis because DNA was not amplifiable by PCR (see Methods).

MTHFR A1298C The frequency of the 1298C polymorphic allele was 34.6% in cases and 29.6% in controls ($P = 0.16$). The *MTHFR* A1298C polymorphism did not significantly affect the risk of ALL in our population in single analysis (OR 0.90, 95% CI 0.36–2.20 and OR 1.07, 95% CI 0.65–1.77 for *MTHFR* 1298CC and 1298AC + CC individuals, respectively). The test for trend did not reach a significant level ($P = 0.20$).

MS A2756G The frequency of the 2756G polymorphic allele was 13.1% in cases and 21.2% in controls ($P = 0.008$). When the *MS* 2756AA genotype was defined as the reference, individuals with *MS* 2756GG genotype showed a 5.0-fold reduction in ALL risk (OR 0.20, 95% CI 0.02–1.45), but it did not reach statistical significance ($P = 0.14$), because of the low representation of the *MS* 2756GG homozygous genotype found in both ALL cases and controls (0.8% and 3.9%, respectively). In fact, data from the more represented AG heterozygous *MS* 2756 genotype gave a significant difference (OR 0.56, 95% CI 0.32–0.90, $P = 0.030$), and computing together the *MS* 2756GG and AG types, a significant ($P = 0.011$) 1.9-fold risk reduction was yielded (OR 0.52, 95% CI 0.30–0.80). The test for trend yielded a significant P value of <0.001.

MTRR A66G The frequency of the 66G polymorphic allele was 47.7% in cases and 53.3% in controls ($P = 0.17$). The *MTRR* A66G polymorphism did not affect significantly the risk of ALL in our population in single analysis (OR 0.71, 95% CI 0.35–1.40 and OR 0.87, 95% CI 0.51–1.46 for *MTRR* 66GG and AG + GG individuals, respectively). The test for trend did not reach a significant level ($P = 0.30$).

Combined Analysis in ALL Cases. Table 2 shows combined results for *MTHFR* 677 and *MS* 2756 polymorphisms. Considering subjects with *MTHFR* 677CC and *MS* 2756AA genotype as the reference, every combination of alleles showed lower than unit OR values, and the statistical significance was reached for large part of them. Anyhow, the evaluation of the combined risks did not differ from the risk estimate obtained by the analysis of the *MTHFR* 677TT genotype alone (Table 1), with the exception of the *MTHFR* 677TT and *MS* 2756AA combination, which yielded a 5.0-fold risk reduction. This leads to the conclusion that also subjects wild-type for both *MTHFR* 677 and *MS* 2756 substitution had an increased susceptibility of 3.6-fold to develop ALL than subjects with at least one polymorphic allele for both variants. Nevertheless, to investigate potential gene-gene interactions between *MTHFR* 677 and *MS* 2756, we applied a logistic regression analysis. Although data could suggest possible interactions, the

analysis did not have sufficient power to detect a significant interaction. The likelihood ratio test for the existence of interaction resulted in a marginal *P* value of 0.098. Combined results for *MTHFR* 677 and *MTRR* 66 polymorphism showed statistical significance only for subjects with *MTHFR* TT and *MTRR* AG genotype, which showed a 4.2-fold ALL risk reduction (OR 0.24, 95% CI 0.06–0.81, $P = 0.040$) when *MTHFR* 677CC and *MTRR* 66AA genotype was considered as the reference. Combined results for *MS* 2756 and *MTRR* 66 polymorphism showed statistical significance only for subjects carrying at least one polymorphic allele for both variants yielding a 2.2-fold ALL risk reduction (OR 0.45, 95% CI 0.10–0.85, $P = 0.045$). Combined results for *MTHFR* C677T and A1298C polymorphism showed statistical significance only for those subjects carrying the 677TT and 1298AA homozygous genotype yielding a 3.0-fold ALL risk reduction (OR 0.33, 95% CI 0.15–0.83, $P = 0.04$). This finding is mainly due to the strong protective effect of the 677TT genotype necessarily present in conjunction with the 1298AA genotype because of the strong negative disequilibrium between the two *MTHFR* polymorphic alleles.

Genotyping and Adjusted Risk Evaluation by Single and Combined Analysis for *MTHFR*, *MS*, and *MTRR* Polymorphisms in NHL Cases. Table 1 shows the allele and genotype frequencies for NHL cases and controls and the adjusted OR values for each polymorphism. The frequencies of the polymorphic variants analyzed were similar in the whole NHL group and in the controls, and all the corresponding OR values were near the unit value for all the polymorphisms investigated. On the other hand, subsetting the whole NHL group ($n = 200$) into low ($n = 102$) and high ($n = 98$) grade of malignancy, the polymorphic *MTRR* G allele was significantly underrepresented in the low-grade cases than in the 257 controls (44.1% *versus* 53.3%, $P = 0.03$) and the *MTRR* GG homozygous genotype gave a risk reduction of 2.0-fold (OR 0.50, 95% CI 0.25–0.99; $P = 0.06$; Table 3). In addition, the combined analysis of *MTRR* 66 with *MS* 2756 polymorphisms was the only one to show appreciable results. In fact, considering those with *MS* 2756AA and *MTRR* 66AA genotype as the reference, the statistical significance was reached for the subjects carrying the *MTRR* 66GG homozygous genotype in combination with the *MS* 2756AA genotype (OR 0.37, 95% CI 0.14–0.85; $P = 0.04$) corresponding to a risk reduction of 2.7-fold. Finally, further subgroup analysis was performed only for diffuse large B-cell lymphoma ($n = 79$) and follicular lymphoma ($n = 53$), respectively, 80.6% of high-grade and 52% of low-grade subgroups. None of the comparisons, single or combined, yielded statistically significant results.

Discussion

In the present study, we analyzed the association between susceptibility to adult ALL or NHL and four polymorphisms in the genes coding for the *MTHFR*, *MS*, and *MTRR* enzymes. Specifically, we found that individuals carrying at least one *MTHFR* substitution at site 677 (C→T) or one *MS* substitution at site 2756 (A→G) showed appreciable reduction in ALL susceptibility. This was particularly evident for subjects carrying either the homozygous 677TT substitution with a decreased ALL risk of 3.6-fold or the homozygous 2756GG substitution with a decreased ALL risk of 5.0-fold. Subjects carrying the 2756GG genotype showed the highest protection because only 1 ALL case among 118 had this genotype. However, the wide 95% CI exceeded the unit value probably because of the relatively small number of GG genotype found in both cases and controls. Heterozygous 677CT or 2756AG genotypes also had a significant ALL risk reduction of about 2-fold. The *MTHFR* 1298 and *MTRR* 66 substitutions did not show significant protection against ALL in single analysis; therefore, we looked at the joint effects between each couple of polymorphisms. In combined analysis for *MTHFR* 677 and *MS* 2756, the risk reduction values were equal to or lower than that of the *MTHFR* TT genotype alone, with the exception of subjects with *MTHFR* 677TT and *MS* 2756AA where the effect was higher (5.0-fold risk reduction). For the other polymorphism combinations, the effects were less pronounced. In fact, when *MTHFR* 677 and *MTRR* 66 were computed together, only the subjects carrying the 677TT with the 66AG genotype showed a 4.2-fold significant risk reduction. Naturally, the protection was not restricted only to those particular classes of genotypes, because other subsets of genotypes might expect

Table 2. Number of ALL patients and controls, sex- and age-adjusted OR and 95% CI, for *MTHFR* C677T and *MS* A2756G polymorphisms

MTHFR 677	*MS* 2756	Cases [*n* (%); *n* = 111]	Controls [*n* (%); *n* = 257]	OR	95% CI	*P*
CC	AA	39 (35.1)	48 (18.7)	1.00	Reference	
CT/TT*	AG/GG*	16 (14.4)	69 (26.8)	0.28	0.14–0.58	<0.001
CC	AG	12 (10.8)	26 (10.1)	0.57	0.25–1.29	0.18
CC	GG	0	4 (1.5)	NE	–	–
CT	AA	39 (35.1)	80 (31.1)	0.61	0.34–1.08	0.09
CT	AG	11 (9.9)	44 (17.1)	0.31	0.14–0.67	0.003
CT	GG	1 (0.9)	4 (1.5)	0.31	0.03–2.87	0.53
TT	AA	5 (4.5)	30 (11.7)	0.20	0.07–0.57	0.003
TT	AG	4 (3.6)	19 (7.4)	0.27	0.07–0.57	0.030
TT	GG	0	2 (0.8)	NE	–	–
CT + TT[c]	AG + GG[c]	72 (64.9)	209 (81.3)	0.42	0.26–0.70	0.001

Note: Test for interaction: $P = 0.098$. NE, not estimated.
*All the subjects carrying at least one variant allele for both polymorphisms in combined way.
[c]All the subjects carrying at least one variant allele for either polymorphisms in combined way.

Table 3. Number of NHL patients (low grade) and controls, sex- and age-adjusted OR and 95% CI, for *MTRR* polymorphism

MTRR A66G	Cases [*n* (%); *n* = 102]	Controls [*n* (%); *n* = 257]	OR	95% CI	*P*
AA	33 (32.3)	59 (22.9)	1.00	Reference	
AG	48 (47.0)	122 (47.5)	0.70	0.41–1.21	0.26
GG	21 (20.6)	76 (29.6)	0.50	0.25–0.99	0.06
AG + GG	69 (67.6)	198 (77.0)	0.62	0.37–1.03	0.08
A	114 (55.9)	240 (46.7)	0.69	0.50–0.96	0.03
G	90 (44.1)	274 (53.3)			

to have greater protection as the double homozygotes (677TT/2756GG or 677TT/66GG), but for both the combinations, no subjects with the double variant were found among ALL cases. Similarly, when *MS* 2756 and *MTRR* 66 were analyzed in combination, only those with at least one variant allele in both genes had approximately a significant 2.2-fold lower susceptibility to develop ALL. In combined analyses, the loss of statistical significance is also due to the small number of patients with rare genotypes, but this observation supports the idea that polymorphic alleles are underrepresented in cases.

Our results on ALL are conceptually in accordance with those recently reported by other groups (16, 29–32). However, Skibola *et al.* (32) did not reach significant association in single analysis for *MS* 2756 but found a potential protective interaction with the serine hydroxymethyltransferase polymorphism against ALL. *MTHFR* 1298 does not seem to significantly affect the risk in our study, either isolated or combined with *MTHFR* 677. This finding contrasts with the hypothesized protective role of the 1298 polymorphic allele found by Skibola *et al.* (29) and Wiemels *et al.* (31) in adult ALL and in molecularly defined childhood leukemia, respectively, and with data from Franco *et al.* (30) in studies on childhood ALL. This could be partially explained by the strong negative disequilibrium found between 677T and 1298C alleles. To our knowledge, no study has been published on the association between *MTRR* 66 polymorphism and hematological malignancies, although data on this polymorphism and other diseases have been reported (24, 35, 36, 38). Results from our analyses on *MTRR* 66 in combination with *MTHFR* 677 and *MS* 2756 suggest a possible involvement of the *MTRR* polymorphism in decreasing ALL risk in the same direction as for the other polymorphisms investigated. In addition, in the subgroup of low-grade NHL, the *MTRR* homozygous 66GG condition was the only one to exhibit a significant 2.0-fold risk reduction, and when joined with the *MS* 2756AA, the risk reduction was higher suggesting a different role of these polymorphisms in ALL and NHL. As in ALL cases, where the combined analysis yielded the highest risk reduction for subjects carrying the *MTHFR* TT genotype with the *MS* AA wild-type, also in NHL, the absence or the very low percentage of combinations for two variants did not allow a complete computing and appreciable risk evaluation.

Very few data are present on lymphoma and folate metabolizing enzymes. Our data on NHL, although nonsignificant or with borderline *P* values, are in contrast with an interesting report of Matsuo *et al.* (16), which observed significant risk reduction for both *MTHFR* polymorphisms but a clearly increased susceptibility for *MS* 2756 in similar subsets of lymphoma. It is noteworthy that the *MS* 2756 genotype distribution in our control population (3.9% GG, 34.6% AG, and 61.5% AA) is comparable with that of Matsuo *et al.* (2.5% GG, 33.3% AG, and 64.2% AA). Therefore, the diverse outcomes should necessarily be ascribable to the lower frequency of the variant allele found in our cases (*e.g.*, 0.8% *versus* 7.2% for the GG genotype, respectively, for our study and Matsuo *et al.*). The reasons for these opposite findings are unclear, although discrepancies may be in part related to differences in the genetic background and/or in features related to the diverse population investigated. Gene-environment interactions, such as diet, chemical exposure, or nutritional intake of folate and related vitamins should also be taken into account. In addition, another important point to consider is that both reports are composed of small numbers of cases and controls warranting the need for larger epidemiological studies.

Altogether, the results of our study are in accordance with the hypothesis that an increased availability of methylene-THF for DNA synthesis and a reduced availability of methionine for DNA methylation may confer protection against ALL (29, 32). The causative mechanisms remain partially unclear. Because the different forms of folate participate in distinct pathways of single-carbon metabolism, several mechanisms may be involved. *MTHFR* C677T determines a reduction in the enzyme activity increasing the pool of methylene-THF and reducing the availability of methyl-THF required for methionine synthesis. Hence, both DNA synthesis and DNA methylation processes might be influenced. The protective effect given by *MTHFR* C677T may be ascribable to a more efficient DNA synthesis due to a reduced rate of uracil misincorporation. The removal of this abnormal base with excision repair processes could provoke DNA double-strand breaks and cause chromosomal instability, translocations, or deletions probably sufficient to originate malignant transformation and clonal expansion (39–41). Alternatively, the lower *MTHFR* activity, reducing the availability of one-carbon donor methyl-THF, could also affect the remethylation of homocysteine to methionine via *MS* enzyme, which is maintained in the activated form by *MTRR*. Polymorphisms within these genes, like *MS* 2756 and *MTRR* 66, affecting the respective enzymatic activities, might act in concert with *MTHFR* polymorphism causing DNA hypomethylation (13) via reduction of *S*-adenosylmethionine levels, the major cellular methyl donor for DNA and RNA methylation. Hypermethylation and

consequent silencing of tumor suppressor genes such as *p15*, *p16*, and *p53* have been established to be important epigenetic mechanisms affecting the genesis of cancer as well as of lymphoid malignancies (42–45). The underrepresentation of the polymorphic variants in our ALL cases suggests that defects in these enzymes and a limited DNA methylation in specific genes might play important roles in ALL risk reduction and is in accordance with a reported growth reduction of human tumor cell lines due to DNA hypomethylation (46).

Methylation status and DNA genetic damage are critical also in lymphomas, although the observed protective effect of the polymorphisms investigated was more strongly evident in leukemia. Apparently, the polymorphisms investigated have different effects in distinct types of cancer, analogous to reports by Skibola *et al.* (29) describing protection against ALL and not for acute myeloid leukemia by *MTHFR* substitutions. Moreover, causative events involved in lymphomagenesis may overcome the protective effects of these polymorphisms as described previously for high alcohol intake in colorectal cancer (25, 28).

In conclusion, our results show that *MTHFR* 677 and *MS* 2756 variant alleles strongly reduce the risk of ALL and suggest a slighter role for *MTRR* and *MS* variants in the risk reduction of both ALL and NHL. These results suggest that these polymorphisms may play a more important role in leukemogenesis than in lymphomagenesis. The means by which they can modulate cancer risk is not yet clearly established. Indeed, we must consider that unfavorable environmental situations such as inadequate folate or micronutrient intakes may act in concert with peculiar genotypes modulating cancer risk by gene-environment interactions (47). Folate assessment is strongly recommended in future studies, because its levels could mask/influence the protective effect of genetic variants. Definitive conclusions should nevertheless be drawn with extreme caution, and further larger epidemiological studies or meta-analyses are required to confirm the present findings.

References

1. Greaves MF. Etiology of acute leukemia. Lancet 1997;349:344-9.
2. Smith MT, Zhang L. Biomarkers of leukemia risk: benzene as a model. Environ Health Perspect 1998;106:937-46.
3. Kuppers R, Klein U, Hansmann ML, Rajyewsky K. Cellular origin of human B-cell lymphomas. N Engl J Med 1999;341:1520-9.
4. Vanesse GJ, Concannon P, Willerford DM. Regulated genomic instability and neoplasia in the lymphoid lineage. Blood 1999;94: 3997-4010.
5. Zingg JM, Jones PA. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. Carcinogenesis 1997;18:869-82.
6. Blount BC, Mack MM, Wehr CM, et al. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage. Proc Natl Acad Sci USA 1997;94:3290-5.
7. Shane B. Folate metabolism. In: Picciano MF, Sokstad ELR, Gregory JF, editors. Folic acid metabolism in health and disease. New York: Willey-Liss; 1990. p. 65-78.
8. Lucock M. Folic acid: nutritional biochemistry, molecular biology, and role in disease processes. Mol Genet Metab 2000;71:121-38.
9. Frosst 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.
10. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R. A second genetic polymorphism in methylenetetrahydrofolate reductase (*MTHFR*) associated with decreased enzyme activity. Mol Genet Metab 1998;64:169-72.
11. Dianov GL, Timchenko TV, Sinitsina OI, Kuzminov AV, Medvedev OA, Salganik RI. Repair of uracil residues closely spaced on the opposite strands of plasmid DNA results in double-strand break and deletion formation. Mol Gen Genet 1991;225:448-52.
12. Stern LL, Mason JB, Selhub J, Choi SW. Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene. Cancer Epidemiol Biomarkers & Prev 2000;9:849-53.
13. Friso S, Choi SW, Girelli D, et al. A common mutation in the 5, 10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. Proc Natl Acad Sci USA 2002;99:5606-11.
14. Leclerc D, Campeau E, Goyette P, et al. A. Human methionine synthase: cDNA cloning and identification of mutations in patients of the cblG complementation group of folate/cobalamin disorders. Hum Mol Genet 1996;5:1867-74.
15. Harmon DL, Shields DC, Woodside JV, et al. Methionine synthase D919G polymorphism is a significant but modest determinant of circulating homocysteine concentrations. Genet Epidemiol 1999; 17:298-309.
16. Matsuo K, Suzuki R, Hamajima N, et al. Association between polymorphisms of folate- and methionine-metabolizing enzymes and susceptibility to malignant lymphoma. Blood 2001;97:3205-9.
17. van der Put NMJ, Gabreels F, Stevens EMB, et al. A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? Am J Hum Genet 1998; 62:1044-51.
18. van der Put NMJ, Steegers-Theunissen RPM, Frosst P, et al. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. Lancet 1995;346:1070-1.
19. Lucock M, Daskalakis I, Hinkins M, Yates Z. An examination of polymorphic genes and folate metabolism in mothers affected by a spina bifida pregnancy. Mol Genet Metab 2001;73:322-32.
20. Gemmati D, Previati M, Serino ML, et al. Low folate levels and thermolabile methylenetetrahydrofolate reductase as primary determinant of mild hyperhomocystinemia in normal and thromboembolic subjects. Arterioscler Thromb Vasc Biol 1999;19:1761-7.
21. Gemmati D, Serino ML, Trivellato C, Fiorini S, Scapoli GL. C677T substitution in the methylenetetrahydrofolate reductase gene as a risk factor for venous thrombosis and arterial disease in selected patients. Haematologica 1999;84:824-8.
22. Szczeklik A, Sanak M, Jankowski M, et al. Mutation A1298C of methylenetetrahydrofolate reductase: risk for early coronary disease not associated with hyperhomocysteinemia. Am J Med Genet 2001; 101:36-9.
23. Chen J, Stampfer MJ, Ma J, et al. Influence of a methionine synthase (D919G) polymorphism on plasma homocysteine and folate levels and relation to risk of myocardial infarction. Atherosclerosis 2001; 154:667-72.
24. Brown CA, McKinney KQ, Kaufman JS, Gravel RA, Rozen R. A common polymorphism in methionine synthase reductase increases risk of premature coronary artery disease. J Cardiovasc Risk 2000;7: 197-200.
25. Ma J, Stampfer MJ, Giovannucci E, et al. Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. Cancer Res 1997;57:1098-102.
26. Slattery ML, Potter JD, Samowittz W, Schaffer D, Leppert M. Methylenetetrahydrofolate reductase, diet, and risk of colon cancer. Cancer Epidemiol Biomarkers & Prev 1999;8:513-8.
27. Shen H, Spitz MR, Wang LE, Hong WK, Wei Q. Polymorphisms of methylene-tetrahydrofolate reductase and risk of lung cancer: a case-control study. Cancer Epidemiol Biomarkers & Prev 2001;10: 397-401.
28. Ma J, Stampfer MJ, Christensen B, et al. A polymorphism of the methionine synthase gene: association with plasma folate, vitamin B12, homocysteine, and colorectal cancer risk. Cancer Epidemiol Biomarkers & Prev 1999;8:825-9.
29. Skibola CF, Smith MT, Kane E, et al. Polymorphisms in the methylenetetrahydrofolate reductase gene are associated with susceptibility to acute leukemia in adults. Proc Natl Acad Sci USA 1999;96:12810-5.
30. Franco RF, Simoes BP, Tone LG, Gabellini SM, Zago MA, Falcao RP. The methylenetetrahydrofolate reductase C677T gene polymorphism decreases the risk of childhood acute lymphocytic leukemia. Br J Haematol 2001;115:616-8.

31. Wiemels JL, Smith RN, Taylor GM, Eden OB, Alexander FE, Greaves MF. Methylenetetrahydrofolate reductase (*MTHFR*) polymorphisms and risk of molecularly defined subtypes of childhood acute leukemia. Proc Natl Acad Sci USA 2001;98:4004-9.
32. Skibola CF, Smith MT, Hubbard A, et al. Polymorphisms in the thymidylate synthase and serine hydroxymethyltransferase genes and risk of adult acute lymphocytic leukemia. Blood 2002;99:3786-91.
33. Gaughan DJ, Kluijtmans LAJ, Barbaux S, et al. The methionine synthase reductase (*MTRR*) A66G polymorphism is a novel genetic determinant of plasma homocysteine concentrations. Atherosclerosis 2001;157:451-6.
34. Ludwig ML, Matthews RG. Structure-based perspectives on B12-dependent enzymes. Annu Rev Biochem 1997;66:269-313.
35. O'Leary VB, Parle-McDermott A, Molloy AM, et al. *MTRR* and *MTHFR* polymorphism: link to Down syndrome? Am J Med Genet 2002;107:151-5.
36. Hobbs CA, Sherman SL, Yi P, et al. Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome. Am J Hum Genet 2000;67:623-30.
37. Wilson A, Platt R, Wu Q, et al. A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida. Mol Genet Metab 1999;67:317-23.
38. Jacques PF, Bostom AG, Selhub J, et al. Effects of polymorphisms of methionine synthase and methionine synthase reductase on total plasma homocysteine in the NHLBI family heart study. Atherosclerosis 2003;166:49-55.
39. Melnyk S, Pogribna M, Miller BJ, Basnakian AG, Pogribny IP, James SJ. Uracil misincorporation, DNA strand breaks, and gene amplification are associated with tumorigenic cell transformation in folate deficient/repleted Chinese hamster ovary cells. Cancer Lett 1999;146: 35-44.
40. Kunz BA. Mutagenesis and deoxyribonucleotide pool imbalance. Mutat Res 1988;200:133-47.
41. Tommerup N, Poulsen H, Brondum-Nielsen K. 5-Fluoro-2′-deoxyuridine induction of the fragile site on Xq28 associated with X linked mental retardation. J Med Genet 1981;18:374-6.
42. Baur AS, Shaw P, Burri N, Delacretaz F, Bosman FT, Chaubert P. Frequent methylation silencing of p15(INK4b) (MTS2) and p16(INK4a) (MTS1) in B-cell and T-cell lymphomas. Blood 1999;94: 1773-81.
43. Nakamura M, Sugita K, Inukai T, et al. P16/MTS1/INK4A gene is frequently inactivated by hypermethylation in childhood acute lymphoblastic leukemia with 11q23 translocation. Leukemia 1999;13:884-90.
44. Guo SX, Taki T, Ohnishi H, et al. Hypermethylation of p16 and p15 genes and RB protein expression in acute leukemia. Leukemia Res 2000;24:39-46.
45. Moller MB, Ino Y, Gerdes AM, Skjodt K, Louis DN, Pedersen NT. Aberrations of the p53 pathway components p53, MDM2 and CDKN2A appear independent in diffuse large B cell lymphoma. Leukemia 1999;13:453-9.
46. Bender CM, Pao MM, Jones PA. Inhibition of DNA methylation by 5-aza-2′-deoxycytidine suppresses the growth of human tumor cell lines. Cancer Res 1998;58:95-101.
47. Rampersaud GC, Kauwell GP, Bailey LB. Folate: a key to optimizing health and reducing disease risk in the elderly. J Am Coll Nutr 2003; 22:1-8.