Methylenetetrahydrofolate Reductase Genotype Affects Risk of Relapse after Hematopoietic Cell Transplantation for Chronic Myelogenous Leukemia

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ABSTRACT

Purpose: Methylenetetrahydrofolate reductase (MTHFR) directs intracellular folate toward homocysteine metabolism and away from nucleotide synthesis. Two common MTHFR polymorphisms, C677T and A1298C, are associated with reduced enzyme activity. We evaluated the association of these polymorphisms with risk of relapse and bcr-abl mRNA transcript detection among 336 Caucasian patients who underwent allogeneic hematopoietic cell transplantation for chronic myelogenous leukemia.

Experimental Design: Data on the transplant course and folate-related exposures were abstracted from medical records. MTHFR C677T and A1298C genotypes were determined using polymerase chain reaction/restriction fragment length polymorphism and TaqMan assays. Qualitative bcr-abl mRNA testing was conducted using a two-step reverse transcription-polymerase chain reaction assay. Cox regression analysis was used to assess the association between MTHFR genotypes and time to relapse and bcr-abl mRNA detection.

Results: A statistically significant decreased risk of relapse was observed in patients with the variant A1298C genotype [1298CC, hazard ratio (HR) = 0.48 and 95% confidence interval (CI) = 0.26–0.88; 1298CC, HR = 0.28 and 95% CI = 0.09–0.84; P-trend < 0.01]. For the joint C677T/A1298C genotype, variant genotypes were associated with a decreased risk of relapse when compared with the wild-type 677CC/1298AA genotype. This risk was lowest for the 677CC/1298CC genotype (HR, 0.23; 95% CI, 0.08–0.72). MTHFR genotypes were not associated with bcr-abl transcript detection.

Conclusions: These findings suggest that individuals with the 677CC/1298AA genotype are at higher risk of relapse after hematopoietic cell transplantation and that the balance of intracellular folate metabolites available for nucleotide synthesis (regulated by the relative activity of the MTHFR enzyme) may affect the progression from bcr-abl positivity to clinical relapse.

INTRODUCTION

As a carrier of methyl groups, folate is an essential nutrient for nucleotide synthesis. Folate deficiency has been shown to induce DNA damage through uracil misincorporation into DNA during replication, leading to an increased risk of DNA double-strand breaks during DNA excision repair and subsequent genetic instability (1, 2). Thus, adequate availability of folate is crucial for rapidly replicating cells such as hematopoietic cells.

Patients undergoing hematopoietic cell transplant (HCT) are at risk of developing folate deficiency due to decreased dietary intake (as a result of oral mucositis, taste changes, nausea, and anorexia), exposure to antifolate chemotherapeutic (methotrexate) and antimicrobial agents (trimethoprim-sulfamethoxazole), and increased folate requirements during regeneration of hematopoietic cells. However, little is known regarding the effect of folate status (environmental and genetic) on treatment outcome in this patient population.

Methylenetetrahydrofolate reductase (MTHFR) catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate and directs the flux of intracellular folate toward the conversion of homocysteine to methionine at the expense of nucleotide synthesis (3). Two common genetic polymorphisms, C677T and A1298C, have been described for this enzyme; both result in amino acid changes, at codons 222 and 429, respectively. The homozygous 677TT genotype, which occurs in approximately 10% to 15% of Caucasian and Asian populations (4), has been shown to have 30% of the MTHFR wild-type enzyme activity in vitro, and the heterozygous (CT) genotype has approximately 60% of wild-type enzyme activity (5). The 1298CC allele has also been found to result in decreased in vitro activity, although to a lesser extent than that seen with the 677TT allele (6, 7). Approximately 5% to 10% of Caucasians carry the 1298CC genotype (8), which results in an enzyme with approximately 90% of wild-type MTHFR activity (9).

The decreased MTHFR activity associated with the variant 677T allele has been shown to result in an accumulation of the folate derivatives needed for nucleotide synthesis (5,10-methyl-
enethyldioleate and 10-formyltetrahydrofolate) at the expense of 5-methyltetrahydrofolate and homocysteine metabolism (10, 11). The variant MTHFR 677T and 1298C alleles have been associated with increased plasma homocysteine levels (9, 12, 13) and decreased risk of certain cancers, especially acute leukemias (14, 15) and colorectal cancer (16, 17).

We reported previously that individuals with the homozygous variant MTHFR 677TT genotype experienced greater oral mucositis and increased time to engraftment after HCT (18). The primary goal of this study was to assess the effect of the MTHFR C677T and A1298C genotypes on risk of relapse after allogeneic HCT among patients with chronic myelogenous leukemia (CML). We hypothesized that folate deficiency would be associated with increased risk of relapse due to increased genetic instability in residual leukemic cells, which may be aggravated by a variant MTHFR genotype. However, in a setting of adequate folate availability, it is also plausible that the increased proportion of folate variants available for nucleotide synthesis in individuals with lower MTHFR activity (due to the variant MTHFR genotypes) will promote genetic stability in any residual leukemic cells and prevent the accumulation of additional chromosomal abnormalities needed for progression to active disease.

In the majority of cases, relapse after HCT involves reactivation of dormant host leukemic cells that managed to survive the conditioning regimen (19). Detection of transcripts of the bcr-abl fusion gene (the cytogenetic abnormality that characterizes CML) has been used to monitor for minimal residual disease. In an attempt to better understand the mechanism underlying any observed association between the MTHFR genotypes and relapse, we therefore evaluated the association between the MTHFR genotypes and detection of bcr-abl mRNA transcripts after transplantation.

MATERIALS AND METHODS

Study Design and Patient Population. Subjects in this retrospective cohort study were patients receiving an allogeneic HCT at the Fred Hutchinson Cancer Research Center (FHCRC) between 1992 and 2002 who (a) had a diagnosis of CML in chronic or accelerated phase before the transplant, (b) were ≥18 years of age at the time of transplant, and (c) were undergoing their first HCT, and (d) received full myeloablative conditioning regimens with either cyclophosphamide/total body irradiation or busulfan/cytosine, as described previously (21, 22). None of the patients received T-cell–depleted bone marrow or peripheral blood stem cell infusions. All patients received methotrexate and cyclosporine for graft-versus-host disease (GVHD) prophylaxis following previously described protocols (23). The study was approved by the FHCRC Institutional Review Board, and all patients provided informed consent.

Data Collection. Medical records and patient databases were used to abstract study data for each study participant. Data collected included height, weight, body surface area, body mass index (kg/m²), age, sex, race, conditioning regimen, average busulfan concentration at steady state, donor relationship, donor sex, date of HCT, number of methotrexate doses, and total dose received post-transplant, leucovorin administration for rescue from methotrexate toxicity, and total dose of trimethoprim-sulfamethoxazole or dapsone for Pneumocystis carinii pneumonia prophylaxis. We also collected data on pretransplant use of multivitamin or folate supplements (yes/no), dates of parenteral nutrition support, and post-transplant use of multivitamin and folic acid supplements. To verify reproducibility of chart abstraction data, 13 charts were selected randomly and reabstracted; complete agreement between the two abstractions was confirmed.

A registered dietitian or dietetic technician collected information on pretransplant dietary supplement use during an initial nutrition assessment visit approximately 2 weeks before HCT. A patient was considered to have used a multivitamin or folate supplement pretransplant if there was documentation of use in either the Nutrition Program records or the medical record.

Laboratory Analyses. Genotyping for the MTHFR C677T polymorphism in the first 223 patients had been performed previously using the polymerase chain reaction (PCR)/restriction fragment length polymorphism method as described elsewhere (18). Genotyping for an additional 113 patients was undertaken using a 5-exonuclease assay and probes with a nonfluorescent quencher (NFQ). PCR reactions consisted of 2.0 μL of 10× TaqMan Buffer A, 4.0 μL of 25 mmol/L MgCl₂, 0.4 μL of 10 mmol/L dATP, 0.4 μL of 10 mmol/L dGTP, 0.4 μL of 10 mmol/L dUTP, 0.2 μL of 10 μmol/L MTHFR 677W probe (5'-VIC-AAATCGCTCCCGCAG-NFQ-3'), 0.2 μL of 10 μmol/L MTHFR 677M probe (5'-FAM-TGAAATCGCTCCCGCA-NFQ-3'), 0.4 μL of 10 μmol/L MTHFR RTAQ primer (5'-CCGAAAGGAGGC- TTTG-3'), 0.4 μL of 10 μmol/L MTHFR RTAQ primer (5'- CGGTGATGCTCCTCAACAA-3'), 0.1 μL of AmpliTaq Gold (5 units/μL), 0.2 μL of AmpErase (1 unit/μL), and 8.9 μL of distilled water. MTHFR A1298C PCR reactions and the TaqMan allele discrimination were performed for the entire study cohort using methods that were identical to those of the MTHFR C677T reactions, with the exception of the use of the MTHFR 1298W probe (5'-VIC-AGTGAAAGGAATGTTTCTTT-NFQ-3'), MTHFR 1298M probe (5'-FAM-AGTGAAAGGAATGTTTCTTT-NFQ-3'), MTHFR 1298RTAQ primer (5'-AGAGCAAA-TGCCCCAAGG- A-3'), and MTHFR 1298RTAQ primer (5'-TTTGGTACCATTC- CCGTTCTT-3'). TaqMan PCR Core Reagent Kit, primers, probes, 384-well optical reaction plates, and optical adhesive covers were all obtained from Applied Biosystems (Foster City, CA).

Amplification was performed on a 384-well GeneAmp PCR System 9700 thermocycler (Applied Biosystems). Cycling conditions for both genotypes followed the TaqMan Universal Thermal Cycling Protocol (50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 s, followed by 60°C for 1 minute). Fluorescence detection and allelic discrimination were performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). All genotyping was performed blinded to patient outcome. Duplication of a random 10% of the genotyping assays yielded 100% concordance.

Time to first positive bcr-abl mRNA test was determined from qualitative bcr-abl data obtained using a two-step reverse transcription-PCR assay as described previously (24, 25). Analysis was restricted to include only tests performed at least 6 months after transplantation because bcr-abl transcripts detected...
during the first 6 months after transplant have been found to be unreliable as indicators of risk of relapse (26–28).

**Outcome Measures.** The FHCRC Long-Term Follow-Up program maintains contact with patients and their referring physicians after discharge from the center and obtains information on relapse and death. Relapse was defined by cytogenetic (≥5 metaphases positive for the Philadelphia chromosome at any point in time or the presence of any Philadelphia chromosomes on two successive cytogenetic evaluations at least 6 months apart; ref. 24) or hematologic criteria.

**Statistical Analysis.** Cox regression models were used to assess the association of MTHFR genotypes with time to relapse. Analyses were performed using SAS version 8.2 (SAS Institute Inc., Cary, NC). A 95% confidence interval (CI) excluding 1.0 or a P of <0.05 was considered statistically significant.

We evaluated the association between MTHFR genotype and relapse for each polymorphic site (C677T and A1298C) and for the combined C677T/A1298C genotype using individuals with the 677CC/1298AA genotype (homozygous wild-type at both loci) as the reference group. Potential confounding factors (including occurrence of GVHD, transplant year, stage of CML at transplant, conditioning regimen, donor relationship, HLA matching status, age, and sex) were evaluated by adding each factor to the unadjusted model and then assessing its effect on the hazard ratio (HR) for each genotype. A time-dependent covariate was generated for the occurrence of GVHD, which defined whether grade 2 or higher acute GVHD or clinical chronic GVHD had occurred before relapse (yes/no). Covariates that altered the HR by at least 10% for one or more of the MTHFR C677T/A1298C genotypes were included in the final model. Only year of transplant met the criteria for inclusion as a covariate in the regression models. Occurrence of GVHD, patient age, sex, stage of CML at transplantation, HLA matching, conditioning regimen, and sex of the donor did not appreciably alter the HR of relapse associated with MTHFR genotype and were not included in the final models.

Exposures affecting folate status, including pretransplant multivitamin or folate supplement use, folic acid supplementation post-transplant, parenteral nutrition support during the intravenous multivitamin shortage (January 1997 to December 1998; ref. 29), transplantation after federally mandated folate fortification of processed grain products in 1998 (30), and total dose of methotrexate or trimethoprim-sulfamethoxazole, were evaluated for their effect on relapse risk and also as potential effect modifiers of the MTHFR-relapse relationship.

Patients who did not relapse were censored at date of death or date of last contact. The Wald test and CIs were used for their effect on relapse risk and also as potential effect modifiers of the MTHFR-relapse relationship.

The majority (86%) of the study population had a diagnosis of CML in chronic phase at the time of HCT. A total of 183 patients (54%) received cytoxan/total body irradiation as their conditioning regimen, and 153 (46%) received busulfan/cytoxan. Busulfan average steady-state plasma concentration of ≥900 µg/L has previously been associated with lower rates of relapse (31). Data on busulfan average steady-state plasma concentration were available for 108 (70%) of the patients who received busulfan/cytoxan conditioning; the majority of whom (n = 93; 86%) had levels of ≥900 µg/L (range, 406-1385 µg/L).

**Use of Supplemental Folate.** Evaluation of supplement use indicated that 107 patients (32%) used either a multivitamin or individual folate supplement before transplant. On initiation of the conditioning regimen, patients at our center are routinely prescribed a daily adult multivitamin supplement containing 400 µg of folate, which they are instructed to continue through the

<table>
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<th>Table 1 Characteristics of the study population (N = 336)</th>
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<td><strong>Demographics</strong></td>
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<td><strong>Transplant information</strong></td>
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<td>Stage of CML at transplant</td>
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<td>Conditioning regimen</td>
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<td>Cytoxan/total body irradiation</td>
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<td>Busulfan/cytoxan</td>
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<td>Source of hematopoietic progenitor cells</td>
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<td>Related donors</td>
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<td>HLA matched</td>
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<td>Unrelated</td>
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<td><strong>MTHFR genotype</strong></td>
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<td>MTHFR C677T</td>
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<td>677CC</td>
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<td>677CT</td>
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* Values represent the mean ± SD (range).

NOTE. Unless otherwise indicated, values represent n (%).
first year post-transplant. Retrospective chart review showed little documented variation from standard practice guidelines.

**MTHFR Genotype Frequencies.** The variant allele frequencies for this study population were consistent with previously published reports in predominantly Caucasian populations (4, 8). None of the study participants was found to have the MTHFR 677CT/1298CC, 677TT/1298AC, or 677TT/1298CC genotypes, which agrees with other reports (6, 7, 32, 33). For completeness, the individual MTHFR C677T and A1298C genotypes were in Hardy-Weinberg equilibrium, as was the combined C677TA1298C haplotype (it is worth noting, however, that this study cohort does not meet the conditions required for Hardy-Weinberg equilibrium because it is a patient group rather than a random sample of the general population).

**Outcomes.** Outcomes for the study cohort are listed in Table 2. Of the 336 study participants, 55 (16%) had relapsed and 93 (28%) had died as of June 2003. The median time to relapse among patients who had relapsed was 370 days. Of the 210 patients who had neither relapsed nor died, 165 (79%) had been contacted within the previous 6 months, and 182 (87%) had been contacted within the past year. Exclusion of patients whose date of last contact was >1 year before data analysis (n = 28) did not significantly alter the HRs associated with risk of relapse, so these patients were included in all data analyses.

*bcr-abl* data were available for 256 patients, of whom 116 (45%) had a *bcr-abl*-positive test at least 6 months post-transplant. The median time to a positive test among patients who received folate supplementation in excess of 400 µg/day in parenteral nutrition solutions (HR, 1.49; 95% CI, 0.71–3.15; n = 97) after adjusting for year of transplant was observed (reference group, 1298AA (wild-type); 1298AC, HR = 0.48 and 95% CI = 0.26–0.88; 1298CC, HR = 0.28 and 95% CI = 0.09–0.84; P-trend <0.01). Fig. 1 shows time to relapse by MTHFR A1298C genotype. Table 3 depicts the hazard of relapse associated with the combined MTHFR C677T/A1298C genotype. Individuals with at least one variant 1298C allele were found to have a decreased risk of relapse compared with the 677CC/1298AA genotype, with a 4-fold reduced risk among those with the 677CC/1298CC genotype (HR, 0.23; 95% CI, 0.08–0.72).

**Folate Status and Risk of Relapse.** No statistically significant associations with relapse risk were observed for total dose of methotrexate or trimethoprim-sulfamethoxazole or for the comparison of patients who underwent transplantation after the initiation of folate fortification in 1998 with those who underwent transplantation before folate fortification. However, there was a statistically nonsignificant increased risk of relapse among patients who used multivitamin supplements before transplant (HR, 1.49; 95% CI, 0.84–2.65; n = 107), among patients who received supplemental folic acid post-transplant (HR, 1.42; 95% CI, 0.34–5.89; n = 11), and among patients who received folate supplementation in excess of 400 µg/day in parenteral nutrition solutions (HR, 1.49; 95% CI, 0.71–3.15; n = 97) after adjusting for year of transplant and MTHFR C677T and A1298C genotype.

**Interactions between Folate Status and MTHFR Genotype.** Pretreatment multivitamin or folate supplement use was found to modify the risk of relapse associated with the MTHFR C677T genotype, but not the A1298C genotype. A statistically nonsignificant decrease in risk associated with multivitamin or folate supplement use pretransplant was observed for individuals with at least one copy of the variant 677T allele (HR, 0.76; 95% CI, 0.30–1.95; n = 58), whereas a statistically significantly increased risk associated with pretransplant multivitamin or folate supplement use was observed for the homozygous wild-type (677CC) individuals (HR, 2.90; 95% CI, 1.27–6.64; n = 49) when compared with individuals with the 677CC genotype who were not taking multivitamin or folate supplements before transplant (P for interaction = 0.04).

**Survival.** No statistically significant associations were detected between the MTHFR genotypes and overall survival (adjusted for age, stage of disease before transplant, and donor relationship).

![Fig. 1](cancerresearch.aacrjournals.org) Cumulative incidence estimates of the probability of relapse by MTHFR A1298C genotype.
**MTHFR Genotype and Risk of Relapse after HCT for CML**

**DISCUSSION**

We observed a trend toward 2- to 4-fold decreased risk of relapse among individuals with the variant alleles of both polymorphisms, which was statistically significant for the variant MTHFR 1298C allele. Individuals with the wild-type C677T and homozygous variant A1298C genotype (677CC/1298CC) had the lowest risk of relapse (HR, 0.23; 95% CI, 0.08–0.72) compared with the wild-type 677CC/1298AA reference group. Although we reported previously (18) that patients with the variant MTHFR C677T genotypes (CT and TT) appear to experience a greater degree of oral mucositis and delayed engraftment early post-transplant, this study suggests that the variant MTHFR 1298C allele is associated with a decreased risk of relapse. Additionally, the association seen with the MTHFR variants was independent of the occurrence of GVHD, stage of CML at transplant, or HLA matching status. Our finding that the MTHFR genotypes were not related to bcr-abl status post-transplant suggest that the balance of intracellular folate metabolites available for nucleotide synthesis (regulated by the relative activity of the MTHFR enzyme) may affect the progression from bcr-abl positivity to clinical relapse.

Retrospective chart review indicated that the average supplemental folate intake met or exceeded estimated folate requirements over the entire treatment course, and thus, the study population was not likely to be folate deficient. Therefore, the findings may be explained through a biological mechanism involving greater availability of 5,10-methylenetetrahydrofolate for nucleotide synthesis among individuals with the variant MTHFR genotype. The higher supply of nucleotides may result in increased genetic stability in any residual leukemic cells and a decreased occurrence of additional chromosomal alterations needed for progression to active disease. The finding that the variant MTHFR genotypes conferred a decreased risk of disease in a setting of adequate folate availability is consistent with previously reported findings in case-control studies of colorectal cancer (17, 34) and acute leukemias (14, 15).

We observed a stronger decrease in risk for the A1298C variant than for the C677T variant allele. This was surprising, given the clear impact of the C677T substitution on enzyme function. The precise biological relevance of the MTHFR A1298C polymorphism is unclear, but its effects have been considered to be less potent than that of the C677T polymorphism (5, 6). For example, whereas the variant 677TT allele has been associated with increased homocysteine levels, the variant 1298C allele does not appear to modify homocysteine levels (6, 7). However, epidemiologic studies of cancer risk suggest that the A1298C polymorphism has relevance in vivo (14, 17).

We also explored whether folate status may modify the associations observed between MTHFR genotype and relapse risk. We identified a statistically significant interaction between pretransplant multivitamin and folate supplement use and the MTHFR C677T polymorphism, with increased risk of relapse among individuals with the MTHFR 677CC genotype who used multivitamin or folate supplements. Although a possible biological mechanism for an interaction between the C677T polymorphism and folate status has been described (35), a limitation of our analysis is that variant CT and TT genotypes had to be combined and that no data on dose or duration of supplement use were available.

There were two events during the study cohort’s treatment time frame that may have modified folate exposure in this patient population. The first was the federally mandated fortification of processed grain products with folic acid, which resulted in an increase in daily folate intake of at least 100 μg per person per day (30, 36, 37). Additionally, from late 1996 through the end of 1998, the United States experienced a shortage of intravenous multivitamin solutions, due to manufacturing...

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**Table 3** Hazard of relapse by MTHFR C677T/A1298C genotype

<table>
<thead>
<tr>
<th></th>
<th>HR (95% CI)</th>
<th>1298AA</th>
<th>1298AC</th>
<th>1298CC</th>
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<tbody>
<tr>
<td>677CC</td>
<td>1.0 (ref.) n  = 39</td>
<td>0.35 (0.14–0.86) n  = 66</td>
<td>0.23 (0.08–0.72) n  = 44</td>
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<tr>
<td>677CT</td>
<td>0.51 (0.23–1.11) n  = 66</td>
<td>0.33 (0.14–0.79) n  = 71</td>
<td>Not observed</td>
<td></td>
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<tr>
<td>677TT</td>
<td>0.49 (0.21–1.16) n  = 50</td>
<td>Not observed</td>
<td>Not observed</td>
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**Table 4** Hazard of bcr-abl mRNA transcript detection (at least 6 months post-transplant) by MTHFR C677T/A1298C genotype

<table>
<thead>
<tr>
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<th>HR (95% CI)</th>
<th>1298AA</th>
<th>1298AC</th>
<th>1298CC</th>
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<tr>
<td>677CC</td>
<td>1.0 (ref.) n  = 27</td>
<td>0.70 (0.36–1.35) n  = 52</td>
<td>0.84 (0.42–1.70) n  = 36</td>
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<tr>
<td>677CT</td>
<td>0.80 (0.42–1.54) n  = 51</td>
<td>0.60 (0.31–1.19) n  = 51</td>
<td>Not observed</td>
<td></td>
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<tr>
<td>677TT</td>
<td>0.81 (0.41–1.60) n  = 39</td>
<td>Not observed</td>
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NOTE. All analyses were adjusted for year of transplant.
issues (29). As a result of the need to combine several individual intravenous and oral vitamin and mineral formulations, our retrospective chart review revealed that patients who required parenteral nutrition support during this time period actually received as much as 1,400 μg of folic acid per day, significantly more than the 400 μg/day normally recommended (38). Because fortification of enriched grain products and the intravenous multivitamin shortage occurred around the same time, it was impossible to differentiate the effects of these events within the study population, and we did not observe statistically significant changes in risk estimates based on these exposures. Year of transplant did modify the hazard estimates of relapse associated with the MTHFR genotypes and was included in all adjusted models as a potential covariate.

In summary, we report that individuals with the MTHFR 677CC/1298CC genotype are at the lowest risk of relapse after transplant compared with individuals with other MTHFR genotypes and that this risk is independent of the occurrence of GVHD. Additional prospective studies assessing total folate and antifolate exposures, including dietary folate intake, are needed before this information could be used to predict treatment outcome or tailor treatment plans. However, given the high prevalence of the wild-type MTHFR genotypes, an increased risk of relapse associated with the wild-type genotypes may be of clinical importance.

ACKNOWLEDGMENTS

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