Methylenetetrahydrofolate Reductase C677T Polymorphism, Folic Acid and Riboflavin Are Important Determinants of Genome Stability in Cultured Human Lymphocytes

Michiyo Kimura,*† Keizo Umegaki,* Mitsuru Higuchi,* Philip Thomas** and Michael Fenech***


ABSTRACT We tested the hypothesis that methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism, folic acid deficiency and riboflavin deficiency, independently or interactively, are important determinants of genomic stability, cell death, cell proliferation and homocysteine (Hcy) concentration in 9-d human lymphocyte cultures. Lymphocytes of seven wild-type (CC) and seven mutant (TT) homozygotes were cultured under the four possible combinations of deficiency and sufficiency of riboflavin (0 and 500 nmol/L) and folic acid (20 and 100 nmol/L) at a constant methionine concentration of 50 μmol/L. Viable cell growth was 25% greater in TT than in CC cells (P < 0.05) and 32% greater at 20 nmol/L folic acid than at 20 nmol/L folic acid (P = 0.002). The comprehensive cytotoxicity-block micronucleus assay was used to measure micronuclei (MNi; a marker for chromosome breakage and loss), nucleoplasmic bridges (NPB; a marker of chromosome rearrangement) and nuclear buds (NBUD, a marker of gene amplification). The MNi levels were 21% higher in TT cells than in CC cells (P < 0.05) and 42% lower in the high folic acid medium than in the low folic acid medium (P < 0.0001). The NBUD levels were 27% lower in TT cells than in CC cells (P < 0.05) and 45% lower in the high folic acid medium than in the low folic acid medium (P < 0.0001). High riboflavin concentration (500 nmol/L) increased NBUD levels by 25% (compared with 0 nmol/L riboflavin) in folate-deficient conditions (20 nmol/L folic acid medium; P < 0.05), and there was an interaction between folic acid and riboflavin that affected NBUD levels (P = 0.042). This preliminary investigation suggests that MTHFR C677T polymorphism and riboflavin affect genome instability; however, the effect is relatively small compared with that of folic acid. J. Nutr. 134: 48–56, 2004.
oughly understand the effect of the MTHFR C677T polymorphism and other metabolic factors that affect the activity of MTHFR on chromosomal instability, an important risk factor in cancer.

To test these various factors we developed an in vitro system of culturing lymphocytes for 9 d with concentrations of micronutrients that are within or close to the physiological range (16–19). We used this system in combination with the cytokinesis-block micronucleus (CBMN) assay in its comprehensive mode (18,19) to measure various markers of genotoxicity and cytotoxicity that are important in assessing the effect of micronutrients on genomic stability and cell death. These markers include 1) micronuclei (MNi), a marker of chromosome breakage and/or loss; 2) nucleoplasmic bridges (NPB), a marker of chromosome rearrangement; 3) nuclear buds (NBUD), a marker of gene amplification; 4) necrosis (NEC); and 5) apoptosis (APOP) (Fig. 2).

We previously used this system to show that MNi, NPB and NBUD levels increase markedly with a decline in folic acid concentration from 120 to 12 nmol/L, which coincides with the physiological range in the serum of individuals consuming an unsupplemented diet (8 to 35 nmol/L) (6,20,21). It is important to note that these markers of genome instability all positively correlate with each other, suggesting that folic acid deficiency affects the generation of breakage-fusion-bridge cycles, which is a hallmark of genomic instability in several types of cancer cells (22). However, we were unable to show that the MTHFR C677T polymorphism affected genomic stability under the conditions of in vitro culture used in that experiment.

We reasoned that the supraphysiological concentrations of methionine (100 μmol/L) and riboflavin (530 nmol/L) in the RPMI 1640 medium might have altered the activity of MTHFR T677T and MTHFR C677C in such a way that differing effects on genome instability between genotypes became indiscernible (17). The range of serum concentrations of methionine in healthy subjects is 20 to 30 μmol/L (23) and that of riboflavin varies from 5 to 50 nmol/L (6). An excess of methionine may increase SAM concentration, which inhibits MTHFR, whereas an excess of riboflavin may increase FAD concentration, which may be expected to promote MTHFR
activity, particularly in the variant enzyme with decreased coenzyme-binding activity (24).

Therefore we repeated these experiments using a physiological concentration of methionine in combination with deficient or adequate concentrations of riboflavin and folic acid. In addition, we measured the homocysteine concentration of the medium to validate the model. The results suggest that 1) the hypothesis that folic acid, riboflavin and MTHFR C677T polymorphism interact to affect chromosomal instability is, to a certain extent, correct, and 2) the in vitro model can be used to study the effect of gene-nutrient interaction on genome stability.

MATERIALS AND METHODS

Recruitment of subjects and genotyping. The study was approved by the Human Experimentation Ethics Committee of CSIRO Health Sciences and Nutrition, Adelaide, Australia. Subjects were recruited from a cohort of volunteers whose genotype for MTHFR C677T, MTHFR A1298C and methionine synthase A2756G was previously determined. The volunteers selected for the study included seven MTHFR 677 TT (mutant type) homozygotes and MTHFR 677 CC (wild type) homozygotes matched for age, gender and the MTHFR A1298C and MS A2756G polymorphisms (Table 1). Genotyping was performed in duplicate using published methods (13,25).

Lymphocyte culture, viability and cell growth assays. Blood (90 mL) was collected from each subject after an overnight fast and before breakfast. The RPMI 1640 culture medium was custom made in the laboratory to achieve the required concentrations of folic acid, riboflavin and l-methionine. All other constituents of the medium were standard for RPMI 1640 as previously described (26), and were all purchased from Sigma (St. Louis, MO). Dialyzed fetal calf serum (FCS; 5%; Trace Biosciences, Victoria, Australia) and 10 kU/L interleukin 2 (Roche Diagnostics, Basel, Switzerland) were added to the medium. The dialyzed FCS contained 356 pmol/L cobalamin and 9 nmol/L folic acid, which equated to 17.8 pmol/L cobalamin and 0.45 pmol/L folic acid in the complete medium. The folic acid concentration of the medium did not change after 3 d of culture. Lymphocytes were cultured at a concentration of 0.5 × 10⁶ viable cells/L. The viable cell counts on d 3, 6 and 9 were used to estimate the cell proliferation rate. An increase in the viable cell count indicates that the proportion of cells completing replicative DNA synthesis and cell division is greater than the proportion of nondividing and/or dying cells. Preliminary dose-response experiments were conducted to determine the range of concentrations of methionine, riboflavin and folic acid that would sustain cell growth over a 9-d period. Each micronutrient was studied individually while maintaining the other micronutrients at their typical concentrations in RPMI 1640 medium.

Cytokinesis-block micronucleus (CBMN) assay. On d 8 a 0.75-mL aliquot of the cell culture was placed in a culture tube and incubated for a further 24 h in the presence of cytochalasin-B (4.5 mg/L). At the end of this period cells were harvested by cyt centrifugation (Shandon; Southern Products, Cheshire, U.K.) and stained using DiffQuik (LabAids, Brisbane, Australia). The slides were coded and scored using established scoring methods and criteria (18). Binucleated cells (BNC; 1000 cells) were scored for the presence of MNI, NBUD and NPB (Fig. 2). The levels of mononucleated, binucleated, multinucleated, apoptotic and necrotic cells were determined by scoring 500 cells. All experiments on each subject were conducted in duplicate, and each experiment on each matched pair was commenced on the same day. The MNI, NBUD and NPB provided a measure of chromosome breakage or loss, chromosome over-replication and gene amplification, respectively. The MNI also provided an indirect measure of genome hypomethylation, because satellite 2 and satellite 3 DNA hypomethylation causes the loss of chromosomes 1, 9 and 16 as MNI (27), and the MNI level is directly correlated with genome hypomethylation (21). Concurrent positive control assays with lymphocytes exposed to γ-radiation (1.5 Gy) were performed for each experiment, yielding MNI levels of 150 to 220 MNI per 1000 BNC. The slides were coded and scored by a single individual who was not aware of the treatment group or genotype of the samples. The Hcy concentration in d-9 medium was measured by fluorescence polarization immunoassay using the Abbott AXSYM system (Abbott Laboratories, Axis-Shield, Oslo, Norway). The coefficient of variation for duplicate measurements was <5%. The Hcy concentration was measured to determine whether the in vitro model replicated the known in vivo effects of MTHFR genotype, folate and riboflavin on homocysteine concentration (6,12,13,15).

Statistical analysis of data. All data were log-transformed before statistical analysis. Results for the various culture media were compared using parametric repeated-measures one-way ANOVA and the Bonferroni multiple-comparison test. Means of two groups were compared using the matched-pair Student’s t-test. Repeated-measures two-way ANOVA was used to determine whether there were significant two-way interactions between folic acid and riboflavin, folic acid and genotype, and riboflavin and genotype, as well as to determine the percentage of total variation attributable to these factors. These analyses, as well as the test for significance between the slopes of the regression lines for the viable cell count dose-response curves, were conducted using Prism 4.0 software (GraphPad, San Diego, CA). Effect size, the ratio of the difference between group means and the SD (28), was also measured. CSS Statistica (Statsoft, Tulsa, OK)

**TABLE 1** Gender, age and genotype characteristics of subjects in this study¹

<table>
<thead>
<tr>
<th>Variable</th>
<th>TT, n = 7</th>
<th>CC, n = 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Female</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Age² (range), y</td>
<td>56.6 ± 8.04</td>
<td>55.14 ± 8.34</td>
</tr>
<tr>
<td>MTHFR 1298 genotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>AG</td>
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<td>CC</td>
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<td>0</td>
</tr>
<tr>
<td>MS 2756 genotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA*</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>AG*</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>GG</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

¹ Abbreviations: CC, wild-type methylenetetrahydrofolate reductase C677T homozygote; MTHFR, methylenetetrahydrofolate reductase; TT, mutant methylenetetrahydrofolate reductase C677T homozygote. Genotypes: AA, wild-type MTHFR A1298C homozygote; AG, MTHFR A1298C heterozygote; CC, mutant MTHFR A1298C homozygote; AA*, wild-type MS A2756G homozygote; AG, MS A2756G heterozygote; GG, mutant MS A2756G homozygote; MS, methionine synthase.

² Values are means ± SD.
was used for three-way ANOVA to identify any significant three-way interactions, ANOVA post hoc to determine the means for each independent variable category (e.g., CC or TT genotype) and Pearson’s test to determine the extent and significance of correlation among all measured variables. Values of $P < 0.05$ were considered significant.

RESULTS

Preliminary experiments showed a clear dose response in cell growth at folic acid concentrations between 4 and 100 nmol/L; 20 nmol/L was the lowest concentration that produced an increase in the number of viable cells. There was also a dose response in cell growth at methionine concentrations between 4 and 100 μmol/L; 16 μmol/L was the lowest concentration that adequately sustained cell growth (Fig. 4A, B). Riboflavin concentrations from 0 to 500 nmol/L did not affect cell growth (Fig. 4C).

Based on the preliminary data, we fixed the methionine concentration at 50 μmol/L, a concentration that is closer to the physiological range and yet not too low that it might compromise cell growth at the lowest tolerable folic acid concentration. The low and high concentrations of folic acid selected were 20 nmol/L (LF) and 100 nmol/L (HF), respectively. These folic acid concentrations are within or close to the physiological range in plasma, which ranges from <7 nmol/L in subjects with a negative folate balance to >50 nmol/L in subjects consuming >400 μg/d of folate (7,8,21). The low and high concentrations of riboflavin selected were 0 nmol/L (LR) and 500 nmol/L (HR), respectively, to provide a deficient dose and a supraphysiological dose [plasma riboflavin concentrations normally range from 5 to 50 nmol/L (6)] and maximize the chances of eliciting an effect on homocysteine or genome stability. We then tested the genome stability, viable cell number and medium Hcy concentration of MTHFR 677 CC and TT lymphocytes cultured for 9 d in each of the four combinations of low and high folic acid and riboflavin media (i.e., LFLR, LFHR, HFLR and HFHR; Table 2). We also calculated the percentage of total variation that could be explained by genotype, folic acid or riboflavin concentration for each variable measured and the effect size of genotype, folic acid and riboflavin concentration on each variable measured (Table 3).

Cell growth was significantly increased in lymphocytes cultured in the HF media and in those with the TT genotype (Tables 2 and 3). Folic acid concentration and genotype accounted for 16.5 and 11.3% of the increase, respectively; however, these two factors did not interact with each other or with riboflavin. To verify that the culture conditions and genotype affected the metabolism and bioavailability of folate, we measured Hcy concentration on d 9. The concentration was significantly decreased by increasing concentrations of folic acid and riboflavin (Table 2). Genotype, folic acid and riboflavin accounted for 8, 10 and 5% of the decrease in Hcy concentration, respectively (Table 3); however, there was no interaction among the three factors.

Levels of the chromosomal stability biomarkers MNi, NBUD and NPB were minimized in the high folic acid media (Table 2). Folic acid accounted for 30, 39 and 12% of the variance in MNi, NBUD and NPB levels, respectively, and genotype accounted for 4 and 11% of the variance in MNi and NBUD levels, respectively (Table 3). The NBUD levels were 27% lower in TT compared with CC cells ($P < 0.002$), whereas MNi levels were 21% higher in TT compared with CC cells ($P < 0.05$). Riboflavin affected only the NBUD levels, which were 25% higher in cells cultured in the LFHR medium than in cells cultured in the LFLR medium ($P < 0.05$) when the data for the CC and TT genotypes were combined (Table 2). Folic acid and riboflavin interacted to affect NBUD levels significantly ($P = 0.042$). Folic acid and riboflavin did not affect apoptosis and necrosis (data not shown). However, there was a marginal (14%) reduction in apoptosis in the TT compared with the CC genotype ($P < 0.10$), with genotype accounting for ~3% of the decrease in apoptosis rate ($P < 0.05$; Table 3).

To analyze the effect of MTHFR C677T polymorphism directly, we combined the data for cells cultured in each of the
four media and compared cells with the CC and TT genotypes cultured under LF (20 nmol/L folic acid) and HF (100 nmol/L folic acid) conditions (Fig. 5A–D). The NBUD level was lower, and the rate of cell growth was greater, in TT cells than in CC cells under both LF and HF conditions. The greater the Hcy concentration was greater in TT cells than in CC cells, but only under LF conditions.

This study measured seven variables related to cell division, genome stability, cell death and folate metabolism. To clarify the interrelationship among these factors, we performed a cross-correlation analysis, as summarized in Table 4. The Hcy concentration correlated positively with each other, and APOP and NEC correlated positively with each other.

### DISCUSSION

Chromosome breakage, loss and rearrangement are important initiating events in cancer; however, they also play an important role during the evolution of cancer when a genome instability phenotype is established (19,29–33). Gene mutations and gene silencing are known to play a critical role in the inactivation of genes involved in DNA repair, in cell cycle control, in the appropriate segregation of chromosomes during mitosis and in apoptosis (34,35), but less is known regarding the effect of dietary factors on the genome instability phenotype. We therefore focused our research on the development of a tissue culture model that may help to predict the effects of diet on the chromosomal stability of human lymphocytes depending on genotype. This could have important applications in the following fields: 1) determining the optimal concentration of micronutrients for genome stability, as a guide to establishing recommended dietary allowances for the prevention of genome damage (4,36), and 2) the development of optimal culture media for the growth of cells required in the biotechnology industry (e.g., cell lines for protein production, stem cells for tissue repair and lymphocytes for cancer immunotherapy).

The folic acid–methionine pathway (Fig. 1) is particularly relevant to the control of genome stability and involves a number of critical enzymes for which several polymorphisms have been identified. In addition, several of these enzymes require vitamins as cofactors; e.g., MTHFR, methionine synthase and serine hydroxymethyltransferase require riboflavin (as a precursor for FAD), cobalamin and pyridoxine, respect-

### TABLE 2

Comparison of genome stability biomarkers, homocysteine and number of viable cells in human lymphocytes grown for 9 d in culture media containing different combinations of folic acid and riboflavin concentrations1,2

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>MTHFR C677T genotype</th>
<th>LFLR</th>
<th>LFHR</th>
<th>HFLR</th>
<th>HFHR</th>
<th>P-value3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>16.9 ± 1.6ab</td>
<td>20.8 ± 3.8a</td>
<td>9.8 ± 2.3b</td>
<td>12.1 ± 1.6b</td>
<td>0.0024</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>23.5 ± 3.4a</td>
<td>22.4 ± 3.4ab</td>
<td>12.2 ± 1.3c</td>
<td>14.2 ± 2.4abc</td>
<td>0.0038</td>
</tr>
<tr>
<td></td>
<td>CC &amp; TT</td>
<td>20.2 ± 2.0a</td>
<td>21.6 ± 2.5a</td>
<td>11.0 ± 1.3b</td>
<td>13.2 ± 1.4b</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>MNi, n/1000 BNC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>32.1 ± 2.4a</td>
<td>38.7 ± 5.2a</td>
<td>19.6 ± 2.7b</td>
<td>20.3 ± 1.9b</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>23.1 ± 3.7a</td>
<td>30.0 ± 3.7a</td>
<td>15.5 ± 1.6b</td>
<td>12.4 ± 1.0b</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>CC &amp; TT</td>
<td>27.6 ± 2.5a</td>
<td>34.4 ± 3.3b</td>
<td>17.5 ± 1.6c</td>
<td>16.4 ± 1.5c</td>
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</tr>
<tr>
<td></td>
<td>NBUD, n/1000 BNC</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>14.6 ± 1.8</td>
<td>15.3 ± 1.9</td>
<td>10.3 ± 1.3</td>
<td>14.0 ± 1.6</td>
<td>0.2268</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>13.6 ± 3.6b</td>
<td>20.3 ± 3.6a</td>
<td>11.1 ± 2.6b</td>
<td>10.5 ± 1.5b</td>
<td>0.0439</td>
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<tr>
<td></td>
<td>CC &amp; TT</td>
<td>14.1 ± 1.7ab</td>
<td>17.8 ± 2.1a</td>
<td>10.7 ± 1.4b</td>
<td>12.3 ± 1.2ab</td>
<td>0.0162</td>
</tr>
<tr>
<td></td>
<td>Hcy, μmol/L</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>CC</td>
<td>1.67 ± 0.15a</td>
<td>1.44 ± 0.09ab</td>
<td>1.36 ± 0.17ab</td>
<td>1.29 ± 0.09b</td>
<td>0.0161</td>
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<tr>
<td></td>
<td>TT</td>
<td>1.93 ± 0.20a</td>
<td>1.74 ± 0.23a</td>
<td>1.67 ± 0.14a</td>
<td>1.39 ± 0.12a</td>
<td>0.0007</td>
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<tr>
<td></td>
<td>CC &amp; TT</td>
<td>1.80 ± 0.12a</td>
<td>1.59 ± 0.11ab</td>
<td>1.51 ± 0.12b</td>
<td>1.34 ± 0.08b</td>
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<td></td>
<td>Viable cells, n = 109/L</td>
<td>0.69 ± 0.05b</td>
<td>0.65 ± 0.05b</td>
<td>0.92 ± 0.07a</td>
<td>0.86 ± 0.06a</td>
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<td></td>
<td>CC</td>
<td>0.86 ± 0.11b</td>
<td>0.83 ± 0.11b</td>
<td>1.13 ± 0.16a</td>
<td>1.09 ± 0.15a</td>
<td>0.0001</td>
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<td></td>
<td>TT</td>
<td>0.77 ± 0.06b</td>
<td>0.74 ± 0.06b</td>
<td>1.02 ± 0.09a</td>
<td>0.98 ± 0.08a</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>CC &amp; TT</td>
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<td></td>
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</tbody>
</table>

1 Values are means ± SEM; n = 7 for CC and TT; n = 14 for CC & TT. Means in a row with superscripts without a common letter differ, P < 0.05.
2 Abbreviations used: BNC, binucleated cell; CC, wild-type methylenetetrahydrofolate reductase C677T homozygote; Hcy, homocysteine; HFHR, high folic acid (100 nmol/L) and high riboflavin (500 nmol/L) culture medium; HFLR, high folic acid (100 nmol/L) and low riboflavin (0 nmol/L) culture medium; LFHR, low folic acid (20 nmol/L) and high riboflavin (500 nmol/L) culture medium; LFLR, low folic acid (20 nmol/L) and low riboflavin (0 nmol/L) culture medium; MNi, micronuclei; MTHFR, methylenetetrahydrofolate reductase; NPB, nucleoplasmic bridge; NBUD, nuclear bud; TT, mutant methylenetetrahydrofolate reductase C677T homozygote.
3 Repeated-measures one-way ANOVA of log-transformed data.
nuclear bud.

Effect of genotype, folic acid and riboflavin on variables measured in human lymphocytes grown in various culture media, expressed as percentage of total variation (V) accounted for by genotype, folic acid concentration and riboflavin concentration and their effect size (ES)\(^1\)–\(^3\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Genotype</th>
<th>Folic acid</th>
<th>Riboflavin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V, %</td>
<td>ES</td>
<td>V, %</td>
</tr>
<tr>
<td>MNi</td>
<td>11.0(^\dagger)</td>
<td>0.59(\dagger)</td>
<td>29.7**</td>
</tr>
<tr>
<td>NBU 1</td>
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<td></td>
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</tr>
<tr>
<td>NBP</td>
<td>0.1</td>
<td>0.05</td>
<td>12.3*</td>
</tr>
<tr>
<td>APOP</td>
<td>2.9*</td>
<td>0.30</td>
<td>0.0</td>
</tr>
<tr>
<td>NEC</td>
<td>0.5</td>
<td>0.12</td>
<td>0.0</td>
</tr>
<tr>
<td>Hcy</td>
<td>7.9*</td>
<td>0.56*</td>
<td>9.91</td>
</tr>
<tr>
<td>Viable cells</td>
<td>11.3*</td>
<td>0.67*</td>
<td>16.51</td>
</tr>
</tbody>
</table>

1 Values determined by two-way ANOVA; *P < 0.05, \(\dagger P < 0.005\), **P < 0.0005.
2 Effect size is expressed as the ratio of the difference between the mean values for the two categories for each independent variable [i.e., genotype, mutant (TT) and wild type (CC); folic acid, high and low concentration; or riboflavin, high and low concentration] and the standard deviation for the dependent variable.
3 Abbreviations: APOP, apoptotic cell; Hcy, homocysteine; MNi, micronuclei; NEC, necrotic cell; NBP, nucleoplasmic bridge; NBUD, nuclear bud.

A novel aspect of the present study is the observation that MTHFR C677T polymorphism interact to determine the chromosomal stability of lymphocytes at physiologically relevant concentrations of folic acid, riboflavin and methionine in vitro. However, the results must be considered with some caution, because the culture conditions may not predict precisely what happens in vivo; the RPMI 1640 medium may be deficient in key micronutrients involved in DNA repair (e.g., zinc) or contain supraphysiological concentrations of other micronutrients involved in the folate–methionine cycle such as choline, the precursor of betaine. The concentration of choline is unlikely to be an important contributor to methionine synthesis in this culture system, because betaine–homocysteine–methyl-transferase is not expressed in lymphocytes (37). The type of folate (i.e., 5-methyltetrahydrofolate or folic acid) might also affect the results, although we previously showed that chromosomal instability does not differ when these two types of folate are compared in the same culture system over the concentration range used in this experiment (38).

The results suggest that folic acid and methionine are two of the key determinants of lymphocyte growth in culture media and that riboflavin does not affect it. In addition, it is evident that the TT genotype provided a significant growth advantage over the CC genotype over the long-term culture period of 9 d. The growth advantage of the TT genotype may be related to reduced cell cycle delay, which may be caused by the nuclear budding process that occurs during S-phase (39), because TT cells express significantly fewer nuclear buds than CC cells. An alternative explanation is the slight reduction in apoptosis rate in TT cells compared with CC cells.

A novel aspect of the present study is the observation that the NBUD level was markedly higher in cells cultured in LFHR medium compared with LFRLR medium, indicating that excess riboflavin may be genotoxic at low folate concentrations. It is important to note that the NBUD level is also the chromosomal instability marker that was most affected by the MTHFR polymorphism (i.e., the NBUD level was lower in TT cells than in CC cells). These data suggest that the mechanism that causes NBUD (a biomarker for gene amplification) under folate-deficient conditions may in fact be aggravated either when the riboflavin concentration is increased or when the CC genotype is present. A high riboflavin concentration may increase the activity of MTHFR, which could cause folate to provide methyl groups for methionine synthesis rather than for thymidylicate synthase. The net result could be increased uracil
FIGURE 6  Mechanistic framework explaining the interrelationship between methylenetetrahydrofolate reductase (MTHFR) genotype, riboflavin (R) and folic acid (F) with respect to 1) CpG methylation and uracil in DNA; 2) aneuploidy and micronuclei (MNi) originating from chromosome loss events; 3) MNi originating from acenric chromosome fragments; nuclear buds (NBUD), nucleoplasmic bridges (NPB) and breakage-fusion-bridge (BFB) cycles; 4) initiation of cancer caused by cytosine-phosphate-guanosine denucleotide (CpG) hypomethylation and aneuploidy; and 5) initiation of cancer caused by increased BFB cycles, MNi originating from acenric chromosome fragments, NBUD and NPB. *For brevity, other carcinogenic mechanisms induced by altered genome methylation, such as silencing of tumor suppressor genes and/or activation of oncogenes, are not included in the diagram.

in the DNA, which could lead to the generation of breakage-fusion-bridge cycles, leading to gene amplification and the removal of amplified DNA by nuclear budding, as explained in our recent reviews (19,22).

One puzzling result of this study is that, although NBUD were markedly modified by genotype (i.e., reduced levels in TT cells), there was no trend toward a reduction of NPB and MNi levels in TT cells compared with CC cells. In fact, MNi levels increased marginally in TT cells. This appears to be counter-intuitive, given that uracil in DNA correlated positively with MNi in our previous studies using this system (16) and in other studies (7,8). However, MNi may originate not only from chromosome breakage caused by uracil in DNA but also from chromosome loss events that may be caused by hypomethylation of DNA (3). The TT genotype increases DNA hypomethylation in lymphocytes in vivo (15), and DNA hypomethylation in lymphocytes in vitro or in vivo increases the loss of chromosomes 1, 9 and 16, which are then included in MNi (40,41). Therefore, the marginal increase in MNi levels in TT cells may be due to increased chromosome loss events.

A possible weakness of our study is that we did not measure DNA methylation directly. However, as indicated above, MNi levels increase under demethylating conditions [e.g., in 5-azacytidine treatment or defects in DNA methyl transferase, as in immunodeficiency, centromeric region instability and facial anomalies (ICF) syndrome (27,42,43) and therefore provide a measure of genome hypomethylation. Furthermore, there is a direct relationship between CpG hypomethylation and MNi expression in vivo (21). In addition, MNi levels under folate-deficient conditions correlate with uracil in DNA, which is another marker of DNA hypomethylation (16). These observations are supported by the tendency shown in our study for MNi levels to increase in MTHFR TT cells that exhibit CpG hypomethylation (15) and increased MNi levels in lymphocytes in vivo (44).

The complexity of the interrelationships among MTHFR genotype, folic acid and riboflavin is illustrated in Figure 6, which provides a succinct mechanistic framework for the results of this study. This mechanistic framework predicts that 1) genome instability from breakage-fusion-bridge cycles and aneuploidy is minimized when folate concentration is increased, 2) high MTHFR activity minimizes genome hypomethylation and aneuploidy caused by chromosome loss or gain at the expense of increased breakage-fusion-bridge cycles and vice versa, 3) high riboflavin concentration in the presence of low folate concentration increases the risk of breakage-fusion-bridge cycles, 4) low riboflavin concentration in the presence of low folate concentration maximizes the risk of genome hypomethylation and aneuploidy caused by chromosome loss or gain, and 5) MNi in MTHFR C677C and MTHFR T677T may not appear to be very different because a low MTHFR activity may decrease MNi caused by uracil and chromosome breakage but also increase MNi originating from chromosome loss or gain caused by CpG hypomethylation and vice versa. These mechanistic interrelationships among MTHFR genotype, folate, riboflavin and genome instability may explain why the MTHFR C677T polymorphism reduces risk for certain cancers, such as leukemia (9), lymphoma (10) and colorectal cancer (11), but increases risk for Down syndrome (12), neural tube defects (13) and cervical cancer (14). We suggest that prevention of chromosome breakage and breakage-fusion-bridge cycles caused by uracil in DNA may be more relevant to the prevention of cancers such as lymphoma and leukemia, whereas prevention of CpG hypomethylation, which may be associated with chromosome loss or gain, could be more relevant to the minimization of risk for cancers caused by integration and expression of parasitic DNA (e.g., human papilloma virus in cervical cancer) and/or cancers caused by aneuploidy (31) and developmental defects caused by aneuploidy, such as Down syndrome.

As noted above, we are uncertain as to how precisely the in vitro culture conditions used in this study reflect conditions in vivo. However, they may constitute a close approximation, because the Hcy concentration data from this study are in good agreement with in vivo data that show an increment in Hcy concentration for the TT genotype, low folic acid and low riboflavin (6,45). Both low folate and MTHFR C677T polymorphism increase plasma Hcy in vivo (46). Our in vitro experiments replicated this effect, showing a clear increment in medium Hcy concentration in the MTHFR TT cells compared with the MTHFR CC cells and an increase with the reduced folic acid concentration. The Hcy concentration also increased moderately with the reduced riboflavin concentration, which corresponds with in vivo data (45). The extent of the total variation in Hcy concentration accounted for by the MTHFR C677T genotype was 7.9%, which is comparable to...
results with in vivo data, i.e., 9.0% of variation accounted for by the MTHFR C677T genotype (47). These data indicate that the in vitro model approximates the in vivo situation quite well. The positive correlation of Hcy concentration with cell proliferation fits well with other studies (48–50), as does the positive correlation with MNi, which is precisely the relationship we have reported in our ex vivo studies in older men (20) and young adults (21). These data and the relatively strong negative correlation of Hcy concentration with apoptosis further support the emerging hypothesis that elevated Hcy concentration is a risk factor for carcinogenic events (20,21,51).

The significant positive correlation among NBP, MNi and NBUD is consistent with our previous studies and provides further evidence that folate deficiency induces breakage-fusion-bridge cycles. The evidence for breakage-fusion-bridge cycles as a key mechanism in cancer may explain not only gene amplification and rapid evolution of the karyotypic abnormalities of cancer but also centrosome abnormalities, which may result when cytokinesis is inhibited by the presence of anaphase bridges induced by chromosome breakage and rearrangement or loss of telomeric DNA (19,30,33). The fact that these events occur at high frequency with moderate folate deficiency within the “normal” physiological range (22) underscores the relevance of folate deficiency as an important risk factor for cancer.

Given that the MTHFR 677 TT homozygotes have a lower risk of developing adult acute lymphocytic leukemia (9) and lymphoma (10), it is important to ask whether any of the biomarkers examined in this study may be potential risk factors for these cancers. It appears from this preliminary data that NBUD is the biomarker most strongly related to the TT genotype, whereas MNi, NBUD and NPB are generally negatively correlated with folic acid, deficiency of which is a risk factor for a variety of cancers (4,11,52). Which of these biomarkers is the strongest predictor of cancer risk should be determined by prospective epidemiological studies; such studies have already linked elevated chromosome aberration rate with cancer risk (53).

In conclusion, this study shows that an in vitro culture system with physiologically relevant concentrations of folic acid, riboflavin and methionine is a valid model for studying the effects of nutrition on genome instability. However, given the relatively small number of subjects in this study, the observed effects of the MTHFR genotype and riboflavin must be considered preliminary. This model can be further improved for use in predicting in vivo events by developing a culture medium that matches the physiological concentrations of all other micronutrients and metabolites found in body fluids. We anticipate that such models can be used to determine the optimal micronutrient concentrations required to minimize genome instability in genetic subgroups and more specifically in individuals.

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LITERATURE CITED


