Associations between Two Common Variants C677T and A1298C in the Methylenetetrahydrofolate Reductase Gene and Measures of Folate Metabolism and DNA Stability (Strand Breaks, Misincorporated Uracil, and DNA Methylation Status) in Human Lymphocytes In vivo

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Abstract

Objective: Homozygosity for variants of the methylenetetrahydrofolate reductase (MTHFR) gene is associated with decreased risk for colorectal cancer. We have investigated the relationships between two variants of the MTHFR gene (C677T and A1298C) and blood folate, homocysteine, and genomic stability (strand breakage, misincorporated uracil, and global cytosine methylation in lymphocytes) in a study of 199 subjects. Results: The frequencies of homozgyosity for the C677T and A1298C variants of the MTHFR gene were 12.6% and 14.6%, respectively. Plasma homocysteine, folate, vitamin B12, 5-methyltetrahydrofolate, and RBC folate were determined in the C677T genotypes. Plasma folate was significantly lower (P < 0.001) in the homozygous variants (6.7 ± 0.6 ng/mL) compared with wild-types (8.8 ± 0.4 ng/mL) and heterozygotes (9.1 ± 0.5 ng/mL). Homocysteine was significantly higher (P < 0.05) in homozygous variants (13.2 ± 1.1 μmol/L) compared with homozygous subjects (10.9 ± 0.4 μmol/L). Homozygous variants had significantly lower (P < 0.05) RBC folate (84.7 ± 6.3 ng/mL) compared with wild-types (112.2 ± 5.2 ng/mL) and heterozygous individuals (125.1 ± 6.6 ng/mL). No significant difference in RBC folate was observed between wild-types and heterozygotes. The A1298C variant did not influence plasma homocysteine, folate, 5-methyltetrahydrofolate, vitamin B12, or RBC folate. Lymphocyte DNA stability biomarkers (strand breaks, misincorporated uracil, and global DNA methylation) were similar for all MTHFR C677T or A1298C variants. Conclusion: Data from this study do not support the hypothesis that polymorphisms in the MTHFR gene increase DNA stability by sequestering 5,10-methylenetetrahydrofolate for thymidine synthesis and reducing uracil misincorporation into DNA. (Cancer Epidemiol Biomarkers Prev 2004;13(9):1436–43)

Introduction

Folate acts as cofactor in numerous biochemical reactions through its ability to donate or accept one-carbon units. Folate deficiency presents a significant health problem. It has been reported, prior to the mandatory fortification of specific foodstuffs with folate in the United States in 1998, that 10% of the adult population and up to 60% of juveniles or elderly in low socioeconomic groups suffered from folate deficiency (1, 2). Moreover, a substantial portion of the population in northern Europe does not consume the recommended folate intake of 200 μg/d (3).

The majority of prospective and case-control studies of serum folate, RBC folate, or reported dietary folate intake are compatible with inverse associations with colon cancer and adenoma risk (4). Folate deficiency may impair DNA synthesis and repair or alter cytosine methylation and gene expression. Folate is fundamental for synthesis of purines and the pyrimidine nucleoside thymidine. dUMP is converted to TMP by thymidylate synthetase using 5,10-methylenetetrahydrofolate as methyl donor. If folate is limiting, the balance of DNA precursors is altered leading to dUMP accumulation and incorporation of uracil into DNA. Under normal conditions, the DNA repair enzyme uracil DNA glycosylase extracts misincorporated uracil from the DNA strand. Subsequent DNA repair enzymes remove the base-free sugar, causing a transient breakage in the DNA molecule that is sealed by DNA ligase. However, if folate availability is continually limited, uracil misincorporation and repair may occur continually in a “catastrophic” repair cycle. Repeated breakage of the DNA molecule...
may ultimately cause chromosomal damage and, in turn, malignant transformation (5-7). However, the human evidence in support of this mechanism is currently limited.

Folate, as 5-methyltetrahydrofolate, is cofactor in the metabolic transformation of homocysteine to methionine. Methionine is metabolized to S-adenosylmethionine (SAM), which methylates cytosine in DNA. DNA methylation controls gene expression. Under conditions of folate deficiency, SAM is limiting, which may lead to DNA hypomethylation, inappropriate proto-oncogene activation and transcription, and malignant transformation (8-10).

Methylenetetrahydrofolate reductase (MTHFR) is a key regulatory enzyme in the metabolism of folate. MTHFR irreversibly converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the primary circulating form of folate. Polymorphisms have been identified in the gene encoding MTHFR (11-13). The two most common variants of the MTHFR gene are located at nucleotides 677 (C677T) and 1,298 (A1298C). These variant genotypes are associated with increased thermolability and substantial diminution of activity of the enzyme in vitro (12, 14). Moreover, homozygosity for either the C677T variant or the A1298C variant is associated with a moderate reduction in the risk for colorectal cancer in some studies (15-17). The mechanisms underlying these associations remain to be established.

In this study, we have investigated the interrelations between MTHFR C677T and A1298C variants and folate, homocysteine, DNA stability, and methylation status in lymphocytes in a population-based sample of subjects who had been selected as controls in a study of colorectal cancer in northeast Scotland.

Materials and Methods

Study Subjects. The present study was carried out in a subset of control subjects who participated in a population-based case-control study of colorectal cancer diagnosed in the period September 1998 to February 2000 in the Grampian region, which includes the city of Aberdeen and surrounding rural areas in northeast Scotland. The controls were randomly selected from Community Health Index for Grampian, a list of everyone who is registered with a general practice in the region and has been shown to have a high level of completeness for the Grampian population (18). Those who declined to participate were replaced. Information on diet and other exposures was obtained by postal questionnaire. Of 670 potentially eligible controls approached, 408 (61%) participated. Subjects were asked to indicate whether they would be willing to take part in further research. Those who responded positively were reapproached and asked to provide a single fasted venous blood sample. Samples were obtained from 199 subjects (100 males and 99 females ages 32–88 years). All subjects gave written informed consent and the Joint Ethical Committee of Grampian Health Board and University of Aberdeen approved the study.

Materials. Ultrapure low melting point and electrophoresis grade standard melting point agarose were from Life Technologies (Paisley, United Kingdom). Frosted microscope slides were from Richardson Supply Co. (London, United Kingdom) and 4',6-diamidino-2-phenylindole was from Boehringer Mannheim (Lewes, United Kingdom). ICN Flow (Irvine, United Kingdom) supplied SimulTRAC Radioassay Kit Vitamin B12 (57Co)/Folate (125I) and Dutch modified RPMI 1640. DNA isolation kits were from Qiagen (West Sussex, United Kingdom). 3H-labeled SAM was from NEN Life Science Products (Hounslow, United Kingdom) and SsI methylase, HindII, and MboII were from New England Biolabs (Hertfordshire, United Kingdom). Uracil DNA glycosylase was obtained from Helena Biosciences (Sunderland, United Kingdom). DS30 Hcy homocysteine assay kits were from Drew Scientific (Barrow-in-Furness, United Kingdom). Expand High Fidelity PCR System was supplied by Roche Diagnostics (East Sussex, United Kingdom). PCR primers for the determination of MTHFR genotypes for polymorphisms C677T and A1298C were from Oswel DNA Service (Southampton, United Kingdom). Deoxynucleotide triphosphate was from Amersham Pharmacia Biotech, Inc. (Buckinghamshire, United Kingdom). FCS was from Globepharm Ltd (Surrey, United Kingdom). LymphoPrep lymphocyte separation medium was supplied by Nycomed (Birmingham, United Kingdom). All other standard chemicals were obtained from Sigma Chemical (Poole, United Kingdom).

Methods. Isolation of Lymphocytes and Preparation of Plasma and Erythrocytes for Measurement of Folate, 5-Methyltetrahydrofolate, Vitamin B12, and Homocysteine. Prior to sampling from the study population, whole blood samples from four laboratory subjects were stored at 4°C for up to 8 hours in the presence or absence of sodium ascorbate (10 mg/0.5 mL blood) to determine the optimum conditions for ensuring folate stability. Plasma total folate and 5-methyltetrahydrofolate concentrations were similar in freshly processed plasma and in plasma isolated after 8 hours at 4°C (9.4 ± 1.8 ng/mL in fresh plasma versus 9.8 ± 1.8 ng/mL after storage for 8 hours for total folate and 24.6 ± 6.4 ng/mL versus 22.5 ± 5.9 ng/mL for 5-methyltetrahydrofolate at 0 and 8 hours, respectively). Addition of ascorbate to the blood prior to isolation did not affect either total plasma or 5-methyltetrahydrofolate (10.4 ± 1 versus 10.3 ± 2.2 ng/mL for total folate at 0 and 8 hours, respectively, and 26.9 ± 7.4 versus 27.1 ± 6.2 ng/mL for 5-methyltetrahydrofolate at 0 and 8 hours, respectively). For the main study, blood was withdrawn from the antecubital vein of each subject into an EDTA-treated Vacutainer and centrifuged at 1,500 × g for 15 minutes at 4°C. The plasma was aliquoted into 1.5 mL plastic tubes (without ascorbate), “snap frozen” in liquid nitrogen, and stored at −80°C for analysis. Blood from all subjects was processed in this way within 4 hours of sampling. The lymphocyte-containing “buffy coat” (~2 mL) was removed and diluted 1:1 with RPMI before layering onto an equal volume of lymphocyte separation medium (specific gravity 1.077 ± 0.001 g/mL) and centrifuged at 700 × g for 30 minutes at 20°C. Lymphocytes were removed into a fresh centrifuge tube, washed in RPMI, and spun for 15 minutes as before. The supernatant was decanted and the pellet was resuspended in RPMI containing 10% heat-inactivated FCS (v/v) before being counted using a Neubauer improved hemocytometer. The cells were spun at 700 × g for 15 minutes at 20°C, resuspended in 90% FCS-10% DMSO (v/v; freezing mix) at a final cell concentration of 1 × 10^6/mL. The cells were then ''snap frozen” in liquid nitrogen and stored at −80°C for analysis.

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density of $3 \times 10^9$/mL, cooled at $-1^\circ$C/min in poly-
styrene, and stored at $-80^\circ$C. Erythrocytes reconstituted to
initial blood volume with PBS following plasma separation
were aliquoted, “snap frozen,” and stored at $-80^\circ$C.

Plasma folate and vitamin B$_12$ were determined using a
commercially available kit [SimulTRAC Radioassay Kit
Vitamin B$_12$ (50°C)/Folate (125)]. Plasma 5-methyltetrahy-
drofolate was measured by high-performance liquid
chromatography with fluorescence detection (19). Be-
cause of conflicting data on the relation between MTHFR
C677T polymorphism and RBC folate levels (20, 21),
which may at least in part be due to a systematic difference
between the results of microbiological and RIA assays by
genotype (21), RBC folate was measured using the RIA kit
as described above (standardized for protein) and by an
adaptation of the Lactobacillus casei microbiological assay
(22). This enables comparison with the literature for either
method of RBC folate assay. Plasma homocysteine was
measured by reverse phase high-performance liquid
chromatography using a D30 Hcy homocysteine assay
kit in combination with a DS50 analyzer.

Samples were reanalyzed if the coefficient of varia-
tion between duplicates was $>10\%$. The coefficient of variation for total plasma folate, total RBC folate, plasma
5-methyltetrahydrofolate, and plasma homocysteine was
3.5%, 6.0%, 6.2%, and 4.9%, respectively.

DNA Isolation and MTHFR Genotyping. DNA was
isolated from lymphocytes using a Qagen DNA Blood
Mini Kit. DNA concentration was determined spectro-
photometrically at 260/280 nm.

Genotype analysis of two common MTHFR variants,
the C677T and the A1298C polymorphisms, was by PCR
followed by RFLP analysis (12). For C677T, the PCR was
carried out in a total volume of 50 µL, containing 0.26 µg
of forward primer and reverse primer, 0.01 µmol/L each
of deoxynucleotide triphosphates (dATP, dCTP, dGTP,
and dTTP), 22 mmol/L Tris-HCl (pH 7.5), 10 mmol/L KCl,
0.1 mmol/L Taq polymerase. The amplification 
conditions were as described by Frosst et al. (12). PCR variables were as
follows: an initial denaturation step of 2 minutes at 94
°C followed by 34 cycles of 94°C for 1 minute (denaturation), 68°C for 1 minute (annealing), and 72°C for 2 minutes
(extension) with a final elongation for 5 minutes at 72°C
to ensure complete extension of all PCR products. The amplified PCR product of 198 bp was digested with the restriction enzyme
MboII (1.25 units per reaction for 2 hours at 37°C) and
analyzed with loading dye (bromophenol blue/xylene
cyanol) on a 10% polyacrylamide gel in a running buffer of
1 x Tris-borate EDTA for 45 minutes at 30 A. DNA was
stained in 150 mL ethidium bromide (0.5 g/mL in water).

Samples that failed on amplification or digest were repeated. Polyacrylamide gels were double read to en-
sure correct identification of variants. Ten percent of all
samples were repeat extracted and PCR was done to certify reproducibility. All 199 subjects were successfully
 genotyped for both polymorphisms.

DNA Strand Breaks and Misincorporated Uracil. DNA
instability (strand breaks and misincorporated uracil) was
measured in lymphocytes isolated from whole blood using
single-cell gel electrophoresis (24, 25). 4',6-Diamino-
dino-2-phenylindole–stained nucleoids were scored vir-
ually using a Zeiss Axioskop fluorescence microscope
(Zeiss, Welwyn Garden City, United Kingdom; ref. 25). One
hundred comet images from each gel (with duplicate
gels per slide) were classified according to the
intensity of fluorescence in the comet tail and assigned
a value of 0, 1, 2, 3, or 4, with 0 representing undamaged
cells and 4 representing maximally damaged cells.
Accordingly, the total score per gel (in arbitrary units)
ranges from 0 to 400. DNA strand breakage was es-
timated based only on the score obtained from buffer-
treated gels. Misincorporated uracil was measured by
subtracting the visual score obtained from buffer-
treated gels from the score obtained after incubation with uracil
dNA glycosylase (25). This method of classification has
been extensively validated using computerized image
analysis (Komet 3.0, Kinetic Imaging Ltd, Liverpool,
United Kingdom; ref. 25). A standard lymphocyte
sample was included with every batch of samples. The
coefficient of variation for the assay was <13%.

Genomic DNA Methylation Status. Total genomic DNA
methylation was determined by measuring incorporation
of methyl groups from $^3$H-labeled SAM at specific cyto-
sine residues using the bacterial enzyme SssI methylase
(26). DNA methylation status is inversely related to the
degree of radioactive incorporation, that is, the lower the
methylation of the DNA, the higher the disintegration
per minute (26). Standard lymphocyte DNA, recovered
and cryopreserved from laboratory volunteers at the
outset of the study, was coanalyzed with each group of
samples. In addition, a single batch of $^3$H-labeled SAM
and SssI methylase was used throughout the study. The
coefficient of variation for the assay was 3.6%.

Statistical Analysis. The genotype frequencies were
assessed to determine whether they were in Hardy-
Weinberg equilibrium. Results obtained for the dietary
biomarkers (plasma folate, homocysteine, 5-methyltetra-
hydrofolate, vitamin B$_12$, and RBC folate measured either
by RIA or microbiologically) and for the DNA stability
biomarkers are presented as mean with range. Log
transforming the data to obtain a normal distribution

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did not affect the overall results, so data are presented untransformed. Differences in levels of the folate biomarkers and measures of DNA stability and methylation status between groups defined by MTHFR genotype were analyzed in SPSS (version 8) by one-way ANOVA followed by Tukey’s HSD test. All results were considered significant if the P of the relevant statistical test was <0.05.

Results

The data were analyzed initially based on either C677T or A1298C genotype without considering status at the second polymorphic site. The frequencies of the homozygosity for the C677T and A1298C variants of the MTHFR gene were 12.6% and 14.6%, respectively (Table 1). Each polymorphism was in Hardy-Weinberg equilibrium. No subject was homozygous for both variants. The vast majority of subjects bearing the 677TT genotype were 1298AA homozygotes. Only two subjects were found bearing a 677TT/1298AC genotype and only one subject was found to have the 677CT/1298CC genotype.

Plasma homocysteine, total folate, vitamin B12, 5-methyltetrahydrofolate, and RBC folate concentrations (measured both by RIA and by microbiological assay) were determined in the C677T genotypes (Table 2). Plasma folate was significantly lower in the homozygous variants (6.7 ng/mL) compared with homozygous wild-type (8.8 ng/mL) and heterozygote individuals (9.1 ng/mL). Similarly, homocysteine was significantly higher in homozygous variants (8.8 ng/mL) compared with homozygous wild-type (7.7 ng/mL) and heterozygote individuals (8.0 ng/mL). No significant difference in plasma folate levels is observed between the various genotypes and DNA stability biomarkers (strand breaks, misincorporated uracil, and 6% of DNA methylation were explained by global cytosine methylation (\( r = 0.07 \) to 0.06; \( P > 0.05 \) in all cases). Moreover, no significant associations were observed between lymphocyte DNA stability biomarkers (strand breaks and misincorporated uracil) or global DNA methylation status and either MTHFR variant (Table 3). The associations between combinations of MTHFR variants and DNA stability and dietary biomarkers were considered. No differences in plasma folate, homocysteine, 5-methyltetrahydrofolate, vitamin B12, and RBC folate levels (measured either by RIA or microbiological assay) were observed between double heterozygotes and the other genotype combinations (data not shown).

Discussion

MTHFR is a key folate metabolizing enzyme, which catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Cancer incidence may be influenced by specific polymorphisms in MTHFR (4). The most extensively researched variant is the C-to-T base transition at position 677 of the gene. Individuals heterozygous (677CT) or homozygous (677TT) for this polymorphism have a reduced in vitro enzyme activity to 65% and 30% of wild-type (677CC), respectively (12). Individuals homozygous for the variant are reported to be at a reduced risk (the relative risks are generally 0.45–0.9) of developing colorectal cancer compared with heterozygotes or wild-types (4, 15, 16). However, the effect of the polymorphism on colorectal cancer risk is significantly influenced by diet and environmental factors, with low folate/methyl donor status and high alcohol intake either negating or reversing cancer risk (reviewed in ref. 4). Moreover, the influence of the TT genotype on cancer risk is site dependent. Whereas homozygous variant individuals seem to be at a reduced risk of developing colorectal malignancy, having the TT genotype is associated with increased prevalence of gastric (27) and cervical dysplasia (28). A second common genetic polymorphism in MTHFR is the A-to-C transition at nucleotide 1,298 (A1298C), which causes a glutamate-to-alanine substitution in the protein associated with reduced enzyme activity in homologotes. Recent reports suggest an inverse association with homoygosity for the 1,298 variant and colorectal cancer (reviewed in ref. 4). The findings that polymorphisms in the MTHFR gene are associated with a decreased risk of

<table>
<thead>
<tr>
<th>Genotype frequency</th>
<th>CC (45.2)</th>
<th>CT (42.2)</th>
<th>TT (12.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1298C</td>
<td>AA (93)</td>
<td>AC (38.7)</td>
<td>CC (14.6)</td>
</tr>
</tbody>
</table>

NOTE: n (%) subjects.
DNA methylation in rodents systems (30). However, the effect of folate deficiency on global or gene-specific DNA methylation is modulating global or gene-specific DNA methylation with evidence demonstrating a role for folate deficiency in vivo. Conversely, the treatment regimen employed (31, 32). Similarly, the relationship between folate deficiency in simple cancer (29). Moreover, DNA hypomethylation is associated with tumor progression and multiplicity in the Apc +/− Min mouse model of colon cancer (29). Moreover, DNA hypomethylation is associated with folate deficiency in simple in vitro cell culture systems (30). However, the effect of folate deficiency on DNA methylation in rodents in vivo is conflicting and highly dependent on the tissue under investigation and the treatment regimen employed (31, 32). Similarly, the evidence demonstrating a role for folate deficiency in colon cancer is contrary to what might be expected given the negative effect of the polymorphism on MTHFR enzyme activity and blood folate levels and the strong inverse association between dietary folate and risk of colorectal cancer.

How polymorphisms in MTHFR influence DNA stability and malignant transformation is at present unknown. MTHFR irreversibly converts 5,10-methylene-tetrahydrofolate to 5-methyltetrahydrofolate, which is crucial in the conversion of uracil to thymidine for DNA synthesis and repair. Expanding intracellular pools of 5,10-methylene-tetrahydrofolate could increase the availability of thymine and thereby enhance DNA stability. In support of this, folate deficiency perturbs intracellular DNA precursor pools and increases uracil misincorporation into DNA. DNA stability is compromised in both human and rodent lymphocytes made folate deficient (25, 36). Moreover, DNA repair is compromised in immortalized human colon cells made folate deficient (30). However, the impact of folate on DNA stability in normal health individuals, not made folate deficient, is less clear.

By studying the influence of variants in the MTHFR gene on folate metabolism, DNA methylation, and uracil misincorporation, the relative importance of these different pathways can be evaluated.

In the present study, homozygous variant individuals (677TT) had significantly higher total plasma homocysteine and lower plasma and RBC folate (measured using the microbial assay) compared with homozygous wild-type (677CC) individuals. This also occurs in other genetic groups (4, 20, 23). Conversely, RBC folate was unaffacted by genotype when measured by RIA, despite a positive correlation between RBC folate measured by both assays and the strong inverse association between dietary folate and risk of colorectal cancer.

### Table 2. Plasma total folate, homocysteine, vitamin B12, 5-methyltetrahydrofolate, and RBC folate stratified by C677T or A1298C genotype

<table>
<thead>
<tr>
<th></th>
<th>677CC (n = 90)</th>
<th>677CT (n = 84)</th>
<th>677TT (n = 25)</th>
<th>1298AA (n = 93)</th>
<th>1298AC (n = 77)</th>
<th>1298CC (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total plasma folate, ng/mL</td>
<td>8.8*</td>
<td>9.1*</td>
<td>6.7</td>
<td>8.6</td>
<td>8.3</td>
<td>8.6 ± 0.71</td>
</tr>
<tr>
<td>Plasma homocysteine, mcg/mL</td>
<td>381.6</td>
<td>379.9</td>
<td>346.9</td>
<td>351.8</td>
<td>397.2</td>
<td>400.8</td>
</tr>
<tr>
<td>Plasma vitamin B12, pg/mL</td>
<td>10.9</td>
<td>11.5</td>
<td>13.2</td>
<td>11.5</td>
<td>11.3</td>
<td>11.9</td>
</tr>
<tr>
<td>RBC folate total, ng/mL</td>
<td>(7.97–9.63)</td>
<td>(8.14–10.03)</td>
<td>(5.43–7.97)</td>
<td>(7.71–9.49)</td>
<td>(7.3–9.3)</td>
<td>(7.89–9.31)</td>
</tr>
<tr>
<td>RBC folate total, ng/mL</td>
<td>(1.35–1.65)</td>
<td>(1.42–1.78)</td>
<td>(1.13–1.67)</td>
<td>(1.34–1.66)</td>
<td>(1.42–1.78)</td>
<td>(1.27–1.73)</td>
</tr>
<tr>
<td>Plasma homocysteine, pmol/L</td>
<td>111.3</td>
<td>116.4</td>
<td>22.6</td>
<td>27.0</td>
<td>22.4</td>
<td>11.9</td>
</tr>
<tr>
<td>Plasma vitamin B12, pmol/L</td>
<td>(10.15–13.65)</td>
<td>(17.74–27.06)</td>
<td>(22.4)</td>
<td>(13.26–21.74)</td>
<td>(19.24–25.96)</td>
<td>(22.4)</td>
</tr>
<tr>
<td>Plasma vitamin B12, pmol/L</td>
<td>(101.33–121.27)</td>
<td>(102.24–133.56)</td>
<td>(22.4)</td>
<td>(19.24–25.96)</td>
<td>(20.42–33.35)</td>
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</tr>
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<td>(19.24–25.96)</td>
<td>(20.42–33.35)</td>
<td>(22.4)</td>
</tr>
</tbody>
</table>

*NOTE: Results are means with 95% confidence intervals.

*P < 0.001, significantly different compared with homozygous variants.

*P < 0.05, significantly different compared with homozygous variants.

DNA methylation is equally inconsistent in humans (33-35). Alternatively, reduced MTHFR activity may increase plasma 5,10-methyltetrahydrofolate, which is crucial in the conversion of uracil to thymidine for DNA synthesis and repair. Expanded intracellular pools of 5,10-methyltetrahydrofolate could increase the availability of thymine and thereby enhance DNA stability. In support of this, folate deficiency perturbs intracellular DNA precursor pools and increases uracil misincorporation into DNA. DNA stability is compromised in both human and rodent lymphocytes made folate deficient (25, 36). Moreover, DNA repair is compromised in immortalized human colon cells made folate deficient (30). However, the impact of folate on DNA stability in normal health individuals, not made folate deficient, is less clear.

### Table 3. DNA stability in lymphocytes (strand breaks and misincorporated uracil) and global DNA methylation status stratified by C677T or A1298C genotype

<table>
<thead>
<tr>
<th></th>
<th>677CC (n = 90)</th>
<th>677CT (n = 84)</th>
<th>677TT (n = 25)</th>
<th>1298AA (n = 93)</th>
<th>1298AC (n = 77)</th>
<th>1298CC (n = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strand breakage, arbitrary units</td>
<td>99.9</td>
<td>111.4</td>
<td>107.2</td>
<td>105.9</td>
<td>109.9</td>
<td>93.8</td>
</tr>
<tr>
<td>Misincorporated uracil, arbitrary units</td>
<td>88.6</td>
<td>82.1</td>
<td>88.1</td>
<td>87.4</td>
<td>81.0</td>
<td>93.0</td>
</tr>
<tr>
<td>Global DNA methylation, disintegration per minute per 0.5 µg DNA</td>
<td>22,805</td>
<td>22,959</td>
<td>23,311</td>
<td>22,892</td>
<td>23,041</td>
<td>22,768</td>
</tr>
<tr>
<td>Misincorporated uracil, arbitrary units</td>
<td>(92.91–106.89)</td>
<td>(101.64–121.16)</td>
<td>(98.2–113.6)</td>
<td>(99.96–119.84)</td>
<td>(82.01–104.59)</td>
<td>(83.01–104.59)</td>
</tr>
<tr>
<td>Global DNA methylation, disintegration per minute per 0.5 µg DNA</td>
<td>(81.74–95.46)</td>
<td>(75.96–88.24)</td>
<td>(71.61–104.59)</td>
<td>(80.6–94.2)</td>
<td>(74.22–87.78)</td>
<td>(80.65–105.35)</td>
</tr>
<tr>
<td>Global DNA methylation, disintegration per minute per 0.5 µg DNA</td>
<td>(22,034–23,876)</td>
<td>(21,428–25,194)</td>
<td>(22,046–23,738)</td>
<td>(22,151–23,931)</td>
<td>(21,386–24,150)</td>
<td>(21,386–24,150)</td>
</tr>
</tbody>
</table>

*NOTE: Results are means with 95% confidence intervals.
problem when comparing data from epidemiologic studies. Individuals homozygous for the C677T polymorphism had significantly elevated homocysteine levels compared with wild-type individuals (677CC), consistent with findings from different populations (37-39). Impaired MTHFR activity with corresponding changes in circulating and cellular folate pools has been proposed to decrease remethylation of homocysteine to methionine. Formylated tetrahydrofolate polyglutamates have been reported to accumulate at the expense of 5-methyltetrahydrofolate species in 677TT subjects (33). In the present study, there was no significant difference in plasma levels of 5-methyltetrahydrofolate in 677CC and 677CT individuals. Failure to observe an effect of genotype on 5-methyltetrahydrofolate concentrations is not due to lack of statistical power, as blood from more than twice the number of 677TT individuals was analyzed in this study compared with that reported previously (40), although it must be noted that different assays were used for analysis (40). No associations between C677T MTHFR genotype and global DNA methylation status were observed. Based on previous studies, DNA hypomethylation due to altered distribution of folate species might have been expected in 677TT individuals compared with 677CC subjects. However, data on the influence of the MTHFR C677T polymorphism on global or site-specific DNA methylation status are inconsistent. One small study found that DNA hypomethylation was increased in leukocytes from 9 677TT individuals compared with 10 subjects with the 677CC genotype (41). In a study of 292 subjects, genomic DNA methylation in peripheral blood mononuclear cells, measured specifically as 5-methylcytosine by liquid chromatography-mass spectroscopy in digested DNA, was lower in 677TT individuals compared with 677CC when plasma folate concentrations were low (42). However, the majority of subjects sampled (66%) had coronary atherosclerosis. Taking into account the association between genotype and global DNA methylation due to altered distribution of folate species, the results of this study may not be generalizable. DNA methylation status in heterozygotes was not measured in either of these studies. Global DNA methylation is decreased in several tissues from MTHFR−/− knockout mice (45). Moreover, SAM levels are depleted, whereas 5-adenosylhomocysteine levels are elevated in tissues from homozygous knockouts (45). Cytosine levels are lower in normal colon, breast, and lung samples from 677CT and 677TT human subjects compared with wild-type individuals (46). However, this association between genotype and global DNA methylation was not seen in tumor samples from the same subjects. Moreover, CpG island methylation status in specific tumor suppressor genes was similar for all variants (46). The similarity in 5-methyltetrahydrofolate levels between subjects with the 677CC, 677CT, or 677TT genotype may partially explain the lack of effect of the MTHFR C677T polymorphism on lymphocyte genomic DNA methylation status in our study. We believe that this is the first study to report the influence of polymorphisms in the MTHFR gene on uracil misincorporation in vivo in a population-based sample not suffering from chronic conditions. Endogenous uracil misincorporation was not significantly related to the C677T genotype in the present study. Homozygosity for the C677T variant (677TT) does not affect the ability of lymphocytes in vitro to resist uracil misincorporation into DNA (47). Similarly, although folate deficiency in vitro induces a dose-dependent increase in chromosomal breakage (measured as micronuclei frequency) and abnormalities in human lymphocytes, this is not influenced by MTHFR genotype (48). Moreover, in a recent human study, endogenous DNA strand breakage, sister chromatid exchange, and micronuclei frequency formation in blood cells were similar for all MTHFR C677T variants (49). DNA methylation and uracil misincorporation were not measured (49). In contrast, micronuclei frequencies have been reported to be elevated in 677TT subjects with coronary artery disease (50). However, as discussed above, DNA damage may be induced by several mechanisms in patients already presenting with disease, which may confound these results. The A1298C variant, like the C677T variant, results in a decrease in MTHFR activity that is more pronounced in the homozygous (1298CC) than in the heterozygous (1298AC) or normal (1298AA) states (23). In contrast with the C677T variant, no associations between A1298C genotype and folate or homocysteine levels were observed in this study. Previous studies have also observed that neither homozygous nor heterozygous individuals have higher homocysteine and/or lower plasma folate levels compared with wild-types (1298AA; refs. 23, 39). Having the C677T variant may have a greater impact on enzyme function; the C677T variant lies within the gene catalytic domain, whereas the A1298C variant is located within the COOH-terminal regulatory domain. DNA stability and methylation status in lymphocytes was not influenced by genotype. It has been suggested that combined heterozygosity for both polymorphisms results in reduced MTHFR activity, higher plasma homocysteine, and lower plasma folate than homozygous wild-types, that is, compound heterozygotes are believed to give a similar metabolic profile as homozygotes for the mutant 677TT polymorphism (51, 52). In this study, compound heterozygotes for both variants did not have significantly altered homocysteine, folate, 5-methyltetrahydrofolate, or vitamin B12 levels compared with any other genotype. These findings agree with previous studies (38, 51, 52). Similarly, DNA stability (DNA strand breaks, misincorporated uracil, or DNA methylation) was not related to compound heterozygosity although, given that only 40 compound heterozygotes were studied, this merits further investigation. The data from this study do not support the hypothesis that polymorphisms in the MTHFR gene increase DNA stability by sequestering 5,10-methylenetetrahydrofolate for thymidine synthesis and reducing uracil misincorporation into DNA. Despite alterations in plasma and RBC total folate levels and a corresponding increase in homocysteine due to genotype, we found no association between MTHFR C677T variants and lymphocyte biomarkers of DNA stability or DNA methylation status. This lack of association between genotype and genomic stability is surprising, given the literature supporting a protective effect of genotype against colorectal cancer and may be partially explained by the
use of peripheral blood cells as a surrogate for the more appropriate and rapidly dividing colonocyte. However, the relative importance of circulating folate levels compared with cellular folate levels in the development of colonic neoplasia remains to be established. Likewise, it is unknown what effect alterations in folate metabolism due to MTHFR polymorphisms would have on uracil misincorporation and DNA synthesis in human colon tissue. Moreover, the influence that polymorphisms in other enzymes of the folate metabolizing pathway may have on genomic stability and cancer risk is unknown. It has recently emerged that MTHFR enzyme activity is profoundly affected by riboflavin (B2). Although the 677TT variant both decreases affinity and increases dissociation of flavin adenine dinucleotide from the enzyme (as cofactor), high levels of flavin adenine dinucleotide can ameliorate this detrimental effect by increasing the availability of flavin adenine dinucleotide for the MTHFR binding site (53-55). However, in a recent study, micronuclei frequency was elevated in human lymphocytes cultured under conditions of low folate as reported previously (47, 48) but was unaffected either by MTHFR genotype and/or riboflavin concentration (56). The influence of riboflavin in vivo on DNA stability in relation to MTHFR genotype was not measured in the present study.

In conclusion, in this study, we found no modulation of endogenous genetic damage by MTHFR polymorphisms. DNA stability and global DNA methylation in human lymphocytes in vitro were not influenced by variants in MTHFR despite significant alterations in folate and homocysteine blood concentrations.

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References

Associations between Two Common Variants C677T and A1298C in the Methylenetetrahydrofolate Reductase Gene and Measures of Folate Metabolism and DNA Stability (Strand Breaks, Misincorporated Uracil, and DNA Methylation Status) in Human Lymphocytes In vivo

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