Short Communication

Genomic DNA Hypomethylation, a Characteristic of Most Cancers, Is Present in Peripheral Leukocytes of Individuals Who Are Homozygous for the *C677T* Polymorphism in the *Methylenetetrahydrofolate Reductase* Gene[1]

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Abstract

DNA methylation is an epigenetic feature of DNA that influences cellular development and function, and aberrations of DNA methylation are a candidate mechanism for the development of cancer. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the synthesis of 5-methyltetrahydrofolate, the methyl donor for methionine synthesis and the precursor of *S*-adenosylmethionine. *S*-adenosylmethionine is the universal methyl donor for methylation reactions, including that of DNA methylation. In the present study, we investigated whether a common *C677T* mutation in the *MTHFR* gene, which results in reduced enzyme activity *in vitro*, affects genomic DNA methylation. We selected 9 subjects homozygous for the wild-type *MTHFR* and 10 subjects homozygous for the mutation (*T/T*). Genomic DNA methylation was determined by an established enzymatic assay that measures the capacity of DNA to accept methyl groups *in vitro*, which is inversely related to endogenous methylation. DNA from subjects with the *T/T MTHFR* genotype had a significantly higher methyl group acceptance capacity (12,615 ± 1836 dpm/2 μg of DNA) compared with wild-type *MTHFR* (7843 ± 1043 dpm/2 μg of DNA; $P < 0.05$), indicating DNA hypomethylation in the *T/T* genotype. Furthermore, DNA methylation was directly and significantly related to RBC folate concentrations in persons with the *T/T* genotype, but not in those with wild-type *MTHFR*. These data are consistent with prior observations, which suggest that the *T/T* genotype is associated with impaired MTHFR activity *in vivo* and that the cellular impact of this impairment is determined, in part, by folate status. The relationship of genomic DNA hypomethylation in persons with the *T/T MTHFR* genotype to the development of cancer remains to be defined.

Introduction

MTHFR[3] [5-methyltetrahydrofolate: (acceptor) oxidoreductase, EC 1.7.99.5] catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methylTHF, the methyl donor for the synthesis of methionine from homocysteine. Methionine is the immediate precursor of SAM, the universal methyl donor for a multitude of biological methylation reactions (1). Included among these essential methylation reactions is DNA methylation, which occurs almost exclusively at CpG dinucleotide repeats in the human genome. This epigenetic modification of DNA has been shown to serve several functional roles, including the suppression of gene expression (2, 3) and the enhancement of genomic stability (4, 5). DNA methylation, therefore, has important effects on cellular development and function. Consistent with this are many observations, which have demonstrated that aberrations in normal patterns of DNA are associated with the development of cancer (6).

Several studies have shown that DNA methylation can be modified by limiting the supply of methyl groups in the diet (7–9), and it is of considerable import that this dietary modification alone is sufficient to produce neoplasms in the liver (10, 11). In a rodent model, a methyl-deficient diet (*i.e.*, combined methionine, choline, and folate deficiency) diminished tissue concentrations of SAM and produced genomic DNA hypomethylation in the liver (7). Gene-specific DNA hypomethylation has also been observed under the condition of folate deficiency alone (8). Furthermore, extended depletion of folate among human subjects in a metabolic ward has been observed to produce genomic DNA hypomethylation (9).

Of increasing interest is a common cytosine-to-thymidine transition in the *MTHFR* gene at nucleotide 677 (*C677T*), which is prevalent in its homozygous form in ~10% of the population (12). This mutation results in an alanine-to-valine substitution in the MTHFR protein and, as shown in *in vitro* studies using cell extracts, renders the enzyme less active and more susceptible to heat inactivation compared with the wild-type enzyme (12, 13).

Data from recent studies suggest that the *in vivo* activity of the thermolabile form of MTHFR is also impaired. As outlined in a review by Rozen (14), persons homozygous for the muta-

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[3] The abbreviations used are: MTHFR, methylenetetrahydrofolate reductase; THF, tetrahydrofolate; SAM, *S*-adenosylmethionine; *T/T*, homozygous for the *C677T* transition in the *MTHFR* gene.

tion (*T/T*) generally have mild elevations in plasma homocysteine concentrations compared with those with wild-type *MTHFR*, particularly when folate status is inadequate. This observation demonstrates how folate status and the *MTHFR* genotype interact to determine the phenotype and implies that persons with the *T/T* genotype synthesize insufficient quantities of 5-methylTHF for the remethylation of homocysteine to form methionine. Indeed, we have recently shown that the *T/T MTHFR* genotype results in a lower proportion of methylated forms of folate in RBCs compared with RBC folates from persons with the wild-type *MTHFR* (15).

The goal of the present study was to assess the impact of the *C677T* mutation on an important cellular process that uses the 5-methylTHF synthesized in the reaction catalyzed by *MTHFR*. We have demonstrated that genomic DNA methylation is diminished in persons homozygous for the *C677T* mutation in the *MTHFR* gene. Furthermore, we investigated whether genomic DNA methylation in persons with the *T/T MTHFR* genotype was related to folate status. This is the first report to date, which demonstrates that the *T/T MTHFR* genotype can alter an epigenetic feature of DNA that has been closely linked to carcinogenesis.

Materials and Methods

Approval for this protocol was obtained from the Tufts-Human Nutrition Research Center Institutional Review Board. Healthy adult volunteers ranging in age from 25 to 75 years were selected according to *MTHFR* genotype. Nine subjects were wild-type (*C/C*) and 10 subjects were homozygous for the *C677T* mutation (*T/T*). Peripheral blood samples were collected after an overnight fast and stored at −70°C until analyses were performed. We determined fasting plasma and RBC folate concentrations in each participant by a conventional microbial assay using *Lactobacillus casei* (16). Fasting total homocysteine concentrations were measured by high-performance liquid chromatography with fluorescence detection (17).

DNA was isolated from peripheral leukocytes using a standard technique (18). Cells were lysed using a buffer containing proteinase K, and DNA was extracted with phenol, chloroform, and isoamyl alcohol. All DNA samples were of high molecular weight (>20 kb), as assessed by gel electrophoresis, and had an A_{260}:A_{280} absorbance ratio >1.8. DNA was frozen at −70°C until used for assay.

MTHFR genotype was determined by PCR-RFLP analysis based on the method described by Frosst *et al.* (12). Briefly, a 198-bp fragment was amplified by PCR using the primers 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ and 5′-AGGACGGTGCGGTGAGAGTG-3′. The presence of the mutant T allele creates a *Hin*fI restriction site resulting in two fragments (175 and 23 bp), the larger of which can be visualized after gel electrophoresis and ethidium bromide staining.

To determine genomic DNA methylation, we used a method that has been detailed previously (19) with minor modifications. This method uses [^{3}H]methyl-SAM and DNA methyltransferase for the *in vitro* transfer of radiolabeled methyl groups to unmethylated sites on DNA. Therefore, DNA with lower methylation *in vivo* will have a greater capacity to accept the radiolabeled methyl groups in this assay. The ability of this method to discriminate between different degrees of *in vivo* DNA methylation has been previously validated (19, 20).

DNA samples (2 μg) were incubated for 2 h at 37°C with 185 kBq [^{3}H]methyl-SAM (20.4 GBq/liter; New England Nuclear, Boston, MA) and 4 units of Sss1 methylase (New England Biolabs, Beverly, MA) in 5 μl of 10× methylation buffer II (New England Biolabs) plus TE buffer [10 mM Tris-HCl, 10 mM EDTA (pH 8.0)] added to a final volume of 50 μl/incubation. The methylation reaction was stopped by heating the samples at 65°C for 20 min. The incubation mixtures were washed onto discs of Whatman DE-81 paper (Fisher Scientific, Springfield, NJ) by using a vacuum filtration apparatus, and then soaked in 50 ml of 0.35 M dibasic sodium phosphate for 30 min. The discs were then dried at 95°C for 30 min, and the resulting radioactivity of the DNA retained in the discs was measured by scintillation counting using a nonaqueous scintillant. All analyses were performed in triplicate.

Mean differences between the wild-type and *T/T MTHFR* genotypes were determined by the Student's *t* test for independent samples. Regression analysis was applied to determine the effect of folate status on genomic DNA methylation. All results were considered significant if the two-tailed observed significance level (*P*) was <0.05. The DNA methylation data were also log-transformed to attain a more normal distribution of the data; however, this transformation did not affect the significance level. Therefore, the results are presented using untransformed data. Values are means ± SD.

Table 1 General characteristics of the study participants according to *MTHFR* genotype[a]

MTHFR genotype	Wild-type	*T/T*
n	9	10
Age	52.2 ± 19.5	48.8 ± 21.1
Male/Female	4/5	4/6
Plasma folate (ng/ml)	10.5 ± 5.5	9.4 ± 4.7
RBC folate (pmol/mg hemoglobin)	2.4 ± 1.1	2.3 ± 1.1
Homocysteine (nmol/ml)	5.5 ± 2.2	6.5 ± 2.6

[a] Values are means ± SD and not different between genotypes (Student's *t* test; $P > 0.05$).

Results

Table 1 shows the general characteristics of the two study groups according to *MTHFR* genotype. The two groups did not differ by age, gender representation, or measures of folate status (*i.e.*, plasma and RBC folate and plasma homocysteine concentrations).

Genomic DNA Methylation. Investigation of genomic DNA methylation showed there was a significant difference between the two *MTHFR* genotypes (Fig. 1). The mean [^{3}H]methyl acceptance capacity of DNA from those with the *T/T MTHFR* genotype (12,615 ± 1836 dpm/2 μg of DNA) was ~40% higher than that of the group without the mutation (7843 ± 1043 dpm/2 μg of DNA; $P = 0.04$). Log transformation of the data likewise resulted in a significant difference ($P = 0.04$). Because methyl acceptance capacity has a reciprocal relationship with DNA methylation, this result demonstrates that the DNA from subjects with the *T/T MTHFR* genotype is hypomethylated compared with the DNA from subjects with wild-type *MTHFR*.

Genomic DNA Methylation and Folate Status. We used regression analysis to determine whether folate status had an effect on genomic DNA methylation. Results showed that as RBC folate decreased, DNA methylation also significantly decreased in those with the *T/T* genotype (genotype-by-RBC folate interaction; $P < 0.02$). Fig. 2 shows the association of RBC folate and [^{3}H]methyl acceptance capacity for each genotype. It is apparent from these graphs that RBC folate was unrelated to DNA methylation in our study subjects with wild-

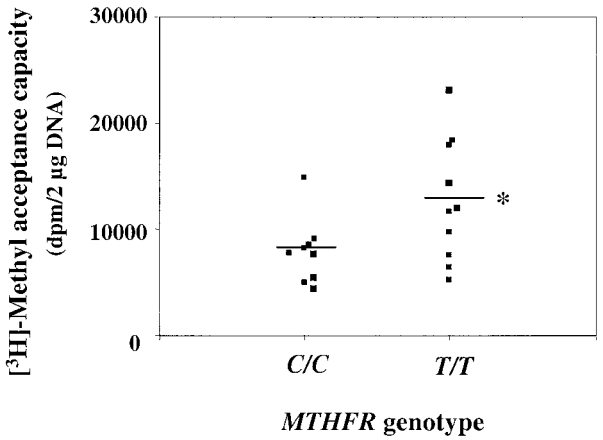

Fig. 1. Genomic DNA methylation. *In vitro* [^{3}H]methyl acceptance capacity was measured in DNA from persons homozygous for the *C677T* mutation in the *MTHFR* gene (*T/T*) and compared with that of persons with wild-type *MTHFR* (*C/C*). The degree of endogenous genomic DNA methylation is inversely related to the incorporation of exogenous tritiated methyl groups. ∗, a significant difference between the two genotypes ($P = 0.04$). The value for each individual is shown (■). *Bars,* mean values for each genotype.

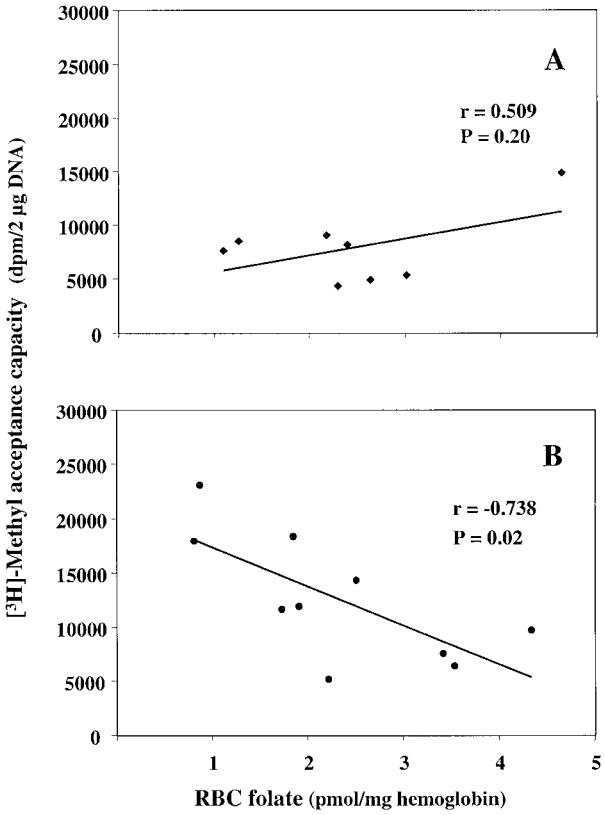

Fig. 2. Genomic DNA methylation and folate status. Linear relationship of [^{3}H]methyl acceptance capacity in DNA to RBC folate concentrations in persons with wild-type *MTHFR* (*A*) and in subjects homozygous for the *C677T* mutation in the *MTHFR* gene (*T/T* genotype; *B*). No correlation was observed in persons with wild-type *MTHFR*; however, there was a significant and inverse association in the *T/T* genotype subjects. One data point is missing because of damage in storage.

type *MTHFR* ($r = 0.509$; $P = 0.20$). However, RBC folate was significantly and inversely correlated to methyl-acceptance capacity (*i.e.,* directly correlated to DNA methylation) in the *T/T* genotype subjects ($r = -0.738$; $P = 0.02$). A similar relationship was observed between plasma folate and DNA methylation in that there was no association of plasma folate and DNA methylation in the wild-type group; however, a strong trend toward diminished DNA methylation was observed in persons with the *T/T* variant with lower plasma folate levels (genotype-by-plasma folate interaction; $P = 0.06$; data not shown). No interaction was observed between *MTHFR* genotype and plasma homocysteine concentrations.

Discussion

The discovery of a common thermolabile variant of the MTHFR enzyme and the demonstration that this variant arises from a *C677T* transition in the structural gene has attracted considerable interest. This interest is primarily attributable to the fact that the *in vitro* activity of the thermolabile enzyme is considerably lower than the normal enzyme (12, 13). Furthermore, this reduction in enzyme activity provides a plausible explanation to early reports showing this mutation is a risk factor for hyperhomocysteinemic-related cardiovascular disease (21, 22), albeit an observation that has not been uniformly reproduced (23–25). Interestingly, the *T/T* genotype appears to protect against carcinogenesis under most conditions. This association of a lower prevalence of the *T/T* genotype in cancer patients compared with healthy controls has been shown particularly in regard to colorectal cancer (26–28) and acute lymphocytic leukemia (29).

An interaction between folate status and the *C677T* mutation has recently been demonstrated, and this has enhanced the interpretation of how this mutation may affect the biochemical measures and clinical outcomes of persons with the *T/T* genotype. For example, Jacques *et al.* (30) showed that persons with the *T/T* genotype and inadequate folate status have significant elevations in plasma homocysteine compared with persons with either the wild-type or heterozygous genotypes. Under the condition of adequate folate status, however, homocysteine levels are low and do not differ between genotypes. This relationship has subsequently been shown by several others (31, 32).

Another important example of this gene-nutrient interaction has been shown in relation to the cancer protective effect of the *T/T* genotype (26–28). These studies reported a ~50% reduction in colorectal cancer risk in both men and women with the *T/T* genotype compared with persons with wild-type *MTHFR*. This protection was no longer evident, however, when blood folate levels or folate intakes were low. In fact, a recent study by Ulrich *et al.* (33) suggests that the *T/T* genotype becomes a risk factor for colorectal adenoma under the condition of low folate intake.

In an attempt to further understand the relationship between folate status and this mutation, Guenther *et al.* (34) recently studied the properties of a thermolabile MTHFR expressed in *Escherichia coli*, which exists in the dimer (inactive) and tetramer (active) forms. These studies showed that the mutation results in a weakening of the association between flavin-adenine dinucleotide and the enzyme, rendering the enzyme more susceptible to disassociation to the inactive dimer form. Folate substrates appear to protect against this inactivation by causing the flavin-adenine dinucleotide molecule to be buried in its binding site and less able to be released from the enzyme. The human MTHFR protein differs from the *E. coli*

enzyme by the fact that it exists only as a dimer and also has an additional peptide region that binds SAM, an allosteric inhibitor of the enzyme. Nevertheless, the studies by Guenther *et al.* (34) did show that folate substrate protects the *E. coli* and human mutant (and normal) enzymes from thermal inactivation.

These *in vitro* studies provide a reasonable interpretation for the epidemiological association between folate status and the *C677T* mutation. However, there still remains to be determined how this interaction is specifically manifested in human cells and tissues. In the present study, we assessed a possible biological effect of this mutation in DNA from peripheral WBCs, a tissue with active folate metabolism. We have shown that persons homozygous for the common *C677T* mutation in the *MTHFR* gene have lower genomic DNA methylation compared with persons with wild-type *MTHFR*. Moreover, we observed that DNA methylation in persons with the *T/T* genotype was directly correlated with RBC folate, a feature that was not seen in the DNA from subjects with wild-type *MTHFR*.

The most reasonable interpretation of these observations is that the activity of the *T/T MTHFR* variant is impaired *in vivo* and that this impairment is dependent on limited substrate availability. Hence, the lower activity of the *T/T MTHFR* variant limits the availability of methyl groups from 5-methylTHF for the synthesis of SAM, thereby reducing the cellular capacity to methylate many biological molecules, including DNA.

A previous study in these same study participants showed that the distribution of folate forms in RBCs was altered as a result of the *T/T MTHFR* genotype (15). Specifically, RBCs from persons with wild-type *MTHFR* exclusively contained methylTHF polyglutamates, whereas RBCs from persons with the *T/T* genotype contained formylTHF polyglutamates in addition to methylTHF polyglutamates. These data suggest there is an impairment on the part of the *T/T MTHFR* variant to convert precursor forms of reduced folates to the methyl derivative. Our present study demonstrates a functional consequence of this reduced availability of methylated folates in the blood cells of persons with the *T/T* genotype.

Our observation of a folate-methylation relationship is entirely consistent with epidemiological evidence suggesting that the activity of the *T/T* genotype MTHFR is further impaired when folate status is inadequate. Notably, we see a graded effect of RBC folate status on genomic DNA methylation in *T/T* genotype individuals without frank folate deficiency. This demonstration that hypomethylation is related to modest reductions in RBC folate, a measurement indicative of tissue folate stores, implies that genomic DNA methylation in peripheral leukocytes may serve as a sensitive and functional biomarker of folate status in persons with the *T/T MTHFR* genotype. The relationships we have demonstrated here are based on a limited number of subjects and therefore need to be confirmed in a larger population. However, despite the limited size of the study population, the relationships are quite robust and are therefore quite provocative.

The potential clinical ramifications resulting from the lower genomic DNA methylation observed in persons homozygous for the *T/T MTHFR* are less clear. Aberrant patterns of DNA methylation are commonly observed in carcinogenesis; diminished genomic and site-specific DNA methylation is an exceedingly common observation (35, 36). Nevertheless, the relevance of our findings to the process of cancer development has yet to be determined. In the case of the *C677T* mutation, the protection against the development of colorectal cancer is thought to be attributable to an enhanced availability of nonmethylated folate substrates for the *de novo* synthesis of purines and thymidylates for DNA synthesis and repair (27). Reports that the *T/T MTHFR* genotype is no longer protective when folate status is low are consistent with the possibility that under this condition, an exaggerated degree of DNA hypomethylation negates the protective effect produced by the increased availability of nucleotides.

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