LETTER TO JMG

5,10-methylenetetrahydrofolate reductase (MTHFR) 677C→T and 1298A→C mutations are associated with DNA hypomethylation

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A growing body of evidence has highlighted the role of abnormal DNA methylation patterns on inappropriate gene expression and promotion of disease.[1–3]

DNA methylation patterns are maintained by DNA methyltransferases,[4–7] using S-adenosylmethionine (AdoMet) as the methyl group donor; AdoMet is then converted to S-adenosylhomocysteine (AdoHcy). Intracellular homocysteine (Hcy) is derived from AdoHcy hydrolysis through the action of AdoHcy hydrolase, a reversible reaction with a dynamic equilibrium that strongly favours AdoHcy synthesis rather than hydrolysis.[8] Thus, an efficient metabolic removal of Hcy is required to prevent AdoHcy accumulation. The toxicity of intracellular AdoHcy accumulation lies in its high affinity binding to the catalytic region of most AdoMet dependent methyltransferases (including DNA methyltransferases), acting as its inhibitor.[9] Thereby, any disturbance in Hcy metabolism is likely to disturb cellular methylation processes, including DNA methylation patterns.

5,10-methylenetetrahydrofolate reductase (MTHFR) is one of the main regulatory enzymes of Hcy metabolism that catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the methyl donor for the remethylation of Hcy to methionine. A common 677C→T transition in the *MTHFR* gene is a well established genetic determinant of hyperhomocysteinaemia, and results in a thermolabile protein, with a decreased enzymatic activity. The molecular basis of this thermolability is a missense mutation in the exon 4 of the *MTHFR* gene, a cytosine to thymine substitution at nucleotide 677, which converts an alanine to a valine codon in the N-terminal catalytic domain of the protein. The association between this *MTHFR* genotype and the total Hcy (tHcy) circulating levels is well known to be contingent on folate status.[10 11]

Recently, a second polymorphism associated with decreased enzymatic activity but not with thermolability was discovered in the *MTHFR* gene.[12] This genetic variant corresponds to an adenosine to cytosine transversion at nucleotide 1298, in exon 7, leading to a glutamate to alanine substitution within the C-terminal regulatory domain of the MTHFR protein. Subjects harbouring the 1298CC genotype have reduced enzyme activity but to a lesser extent than those bearing the 677TT genotype, probably because of the distinct locations of the two polymorphisms; the 677C→T and 1298A→C mutations are found in regions encoding the N-terminal catalytic and the C-terminal regulatory domains of the protein, respectively.[13] The effects of the 1298A→C mutation on plasma concentrations of tHcy remain controversial; we have recently reported that this *MTHFR* mutation displayed a significant effect on plasma tHcy levels,[14] but others have either not found any effect[12 15] or have found an association with even lower levels of plasma tHcy in homozygous individuals.[16]

Key points

- We investigated the effect of the common 677C→T and 1298A→C 5,10-methylenetetrahydrofolate reductase (MTHFR) mutations on leucocyte genomic DNA methylation status.
- The results showed that the mutant 677TT genotype was associated with significantly decreased DNA methylation status. Mutant 1298CC homozygotes displayed lower DNA methylation status, but the difference only reached significance in the absence of the 677C→T mutation—that is, when compared with the double wild-type genotype 677CC/1298AA.
- We conclude that both MTHFR mutations, when in homozygous state, showed effects on DNA methylation status, although these were slightly less pronounced for the 1298A→C transversion.
- Our data suggest that the 1298CC MTHFR genotype, independently of folate availability, and the 677TT MTHFR genotype with concomitant folate inadequate levels, might be potential risk factors of disease states associated with DNA hypomethylation status.

Although it was recently reported that the 677C→T transition in the *MTHFR* gene affects genomic DNA methylation,[3 17] the effect of the 1298A→C transversion has not been evaluated so far. This study is aimed at evaluating, in a healthy population, the effect of both *MTHFR* mutations on the genomic DNA methylation status of white blood cells, taking into consideration the potential influence of folate status.

METHODS

We studied 96 healthy unrelated white Portuguese subjects, 59 women and 37 men, aged 20–69 years (mean age, 40; standard deviation, 14), recruited among staff and students of the Faculty of Pharmacy, and whose characteristics are described in detail elsewhere.[14] The local ethical committee approved the study and a written informed consent was obtained from all participants.

After overnight fasting (12 hours) blood samples were drawn by venipuncture. Blood was collected either into tubes containing EDTA and kept on ice, or into tubes containing sodium citrate and protected from light. Blood collected into

Abbreviations: 95% CI, 95% confidence intervals; AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; Hcy, homocysteine; MTHFR, 5,10-methylenetetrahydrofolate reductase; tHcy, total homocysteine

EDTA was used for tHcy and routine biochemical parameters measurements and for genomic DNA preparation. Blood collected into sodium citrate was used to determine haematocrit, plasma, and red blood cell folate concentrations. Plasma was promptly separated by centrifugation at 4℃, divided into aliquots and stored at −20℃ until analysis. Genomic DNA was extracted from white blood cells using standard phenol and chloroform procedures,[10 12 14 18–21] and then stored at 4℃ until genetic analysis and global DNA methylation quantification. Plasma tHcy (protein bound plus free oxidised and reduced species) and plasma folate, as well as red blood cell folate concentrations, were determined by specific immunoassays (IMX, Abbott Laboratories, Abbott Park, IL, USA).

The *MTHFR* gene mutations, 677C→T and 1298A→C, were analysed by polymerase chain reaction/restriction fragment length polymorphism as previously described.[10 12 14 20 21]

All the DNA samples had a high molecular weight (>20 kb) and an A260/A280 absorbance ratio ⩾1.8. Assessment of the global DNA methylation status was accomplished using the cytosine extension assay as described in detail elsewhere.[22] All analyses were performed in duplicate. The radiolabel incorporation was proportional to the number of unmethylated (cleaved) sites in DNA; thus an increase in relative [^{3}H]-dCTP incorporation reflects hypomethylation. All samples were analysed in four consecutive series of experiments. In each series, four samples from the previous series were randomly selected, and again processed to determine the DNA methylation status. The coefficient of variation between the [^{3}H]-dCTP incorporation obtained in the two measurements was always <10%. This method for discriminating different degrees of in vivo DNA methylation has been previously validated.[17 22]

Vitamin and tHcy concentrations and DNA methylation status were expressed as median values and 95% confidence intervals (95% CI). These values were positively skewed and a logarithmic transformation was performed. One way analysis of variance was used to assess the differences in continuous variables between different genotypes. Whenever the results of the one way analysis of variance were positive, the differences between mean values were detected by Student's *t* test corrected for multiple comparisons (Bonferroni-Dunn test). For all statistics, p values are two tailed and significance was accepted at the 5% probability level. SPSS 10.0 (Chicago, USA) and EPI–Info 2000 (CDC, Atlanta, USA) software packages were used for analysis.

RESULTS

The separate effect of each *MTHFR* genotype (677C→T or 1298A→C) on the global DNA methylation status was studied. Subjects were divided into three groups according to their independent status relative to each of the two studied polymorphic sites (table 1). In what concerns the 677C→T transition, the individuals homozygous for the 677T allele displayed significantly higher ($p<0.05$) relative [^{3}H]-dCTP incorporation than those harbouring the wild-type genotype (table 1 and fig 1A). Thus, the mutant 677TT genotype was associated with decreased leucocyte DNA methylation status. Concerning the effect of the 1298A→C transversion in the *MTHFR* gene on genomic DNA methylation status, the results revealed that mutant homozygotes (1298CC) displayed higher relative [^{3}H]-dCTP incorporation than those with the 1298AA wild-type genotype, but the difference was not statistically significant (table 1 and fig 1B).

All subjects were further stratified taking into account both *MTHFR* mutations simultaneously and six different genotypes were observed (fig 2). In accordance with previous reports, the rare 677TT/1298AC, 677CT/1298CC, and 677TT/1298CC genotypes[23] were not observed in the studied population. Subjects with a 677TT/1298AA or 677CC/1298CC genotype displayed significantly higher ($p<0.05$) values (26.3×10^3 dpm/μg DNA, 95% CI 22.2–37.6 and 25.8×10^3 dpm/μg DNA, 95% CI 22.8–27.1, respectively) for relative radiolabel incorporation than the ones with the double wild-type genotype 677CC/1298AA (19.9×10^3 dpm/μg DNA, 95% CI 17.0–22.2). Subjects carrying the double heterozygous genotype (677CT/1298AC) displayed values (21.3×10^3 dpm/μg DNA, 95% CI 19.1–23.7) similar to those bearing a single heterozygous genotype, either the 677CT/1298AA (21.3×10^3 dpm/μg DNA, 95% CI 18.6–22.4) or the 677CC/1298AC (21.7×10^3 dpm/μg DNA, 95% CI 20.4–23.8) genotypes, which although slightly higher were not significantly different from those carrying the double wild-type genotype 677CC/1298AA.

To assess possible associations between the relative radiolabel incorporation and plasma tHcy levels, or plasma and red blood cell folate levels, regression analyses were carried out either in all subjects together, or in subjects divided into three groups based on their independent status relative to each of the two studied *MTHFR* polymorphic sites (677C→T transition or 1298A→C transversion); no significant associations were found (data not shown). However, significant associations were found between plasma tHcy and the DNA methylation status, in individuals bearing the 677TT genotype and plasma folate levels under the median ($r = 0.87$; $p<0.05$, $n = 6$), or red blood cell folate levels above the median ($r = 0.97$; $p<0.05$, $n = 5$).

DISCUSSION

The present study shows that genomic DNA from healthy subjects bearing the 677TT *MTHFR* genotype was hypomethylated when compared with subjects bearing the wild-type genotype, in accordance with previous reports.[3 17] Furthermore, when the three different 677C→T genotypes were stratified according to their plasma or red blood cell folate levels, significant associations were found between plasma tHcy levels and DNA methylation status, but only in those with the 677TT genotype and either plasma folate levels under the median or red blood cell folate levels above the median. This observation corroborates the concept that mutant 677C→T MTHFR activity is mediated by folate availability, leading to increased tHcy level in the presence of inadequate folate levels. The apparent discrepancies of the effect of the mutant *MTHFR* genotype on red blood cell folate levels may be accounted for by the different methods used for their measurements.[14 24] In fact, we[14] and others[12 15] observed higher red blood cell folate levels in 677TT subjects when compared with the wild-type genotype, whereas Friso et al[3] reported decreased levels.

Regarding the effect of the 1298A→C transversion in the *MTHFR* gene on genomic DNA methylation status, it was observed that homozygotes for this mutation displayed lower DNA methylation status, comparable with the 677TT genotype. However, the difference only reached significance in the absence of the 677C→T mutation—that is, when compared with the double wild-type genotype 677CC/1298AA (fig 2). This observation is in agreement with the fact that the 1298A→C *MTHFR* transversion also reduces MTHFR activity, although to a lesser extent than the 677C→T *MTHFR* transition. When the three different 1298A→C genotypes were stratified according to their folate status, no associations were found between plasma tHcy levels and [^{3}H]-dCTP incorporation. This observation suggests that folate status is not a strong mediator of the 1298A→C MTHFR activity.

For both studied mutations, the presence of a single mutant allele was associated with only a slight decrease in the DNA methylation status, even when double heterozygosity was present, showing no additive effect (fig 2).

Table 1 *MTHFR* genotypes and concentrations of plasma total homocysteine and folate, red blood cell folate, and [^{3}H]-dCTP incorporation

***MTHFR* 677C→T genotype**	**C/C (n = 44)**	**C/T (n = 43)**	**T/T (n = 9)**
Homocysteine (μM)	8.3 (7.9–9.2)	9.0 (8.3–9.8)	10.2 (8.9–12.1)*
Plasma folate (nM)	16.6 (16.7–21.6)	15.9 (13.9–20.2)	12.5 (10.0–17.9)*
Red blood cell folate (nM)	616 (520–632)	555 (479–608)	688 (568–933)*
[^{3}H]-dCTP incorporation (10^3 dpm/μg DNA)	21.8 (20.2–23.0)	21.3 (19.8–24.0)	26.3 (22.2–37.6)*
***MTHFR*1298A→C genotype**	**A/A (n = 48)**	**A/C (n = 41)**	**C/C (n = 7)**
Homocysteine (μM)	9.1 (8.20–9.5)	8.2 (8.1–9.5)	10.5 (9.3–12.4)*
Plasma folate (nM)	15.4 (14.9–20.6)	16.8 (15.0–20.4)	16.5 (13.1–19.4)
Red blood cell folate (nM)	618 (546–694)	580 (527–772)	734 (467–890)
[^{3}H]-dCTP incorporation (10^3 dpm/μg DNA)	21.7 (20.3–25.0)	21.9 (20.3–23.1)	25.8 (22.8–27.1)

All values are expressed as median and 95% confidence intervals.
*p<0.05 for 677TT versus 677CC or for 1298CC versus 1298AA

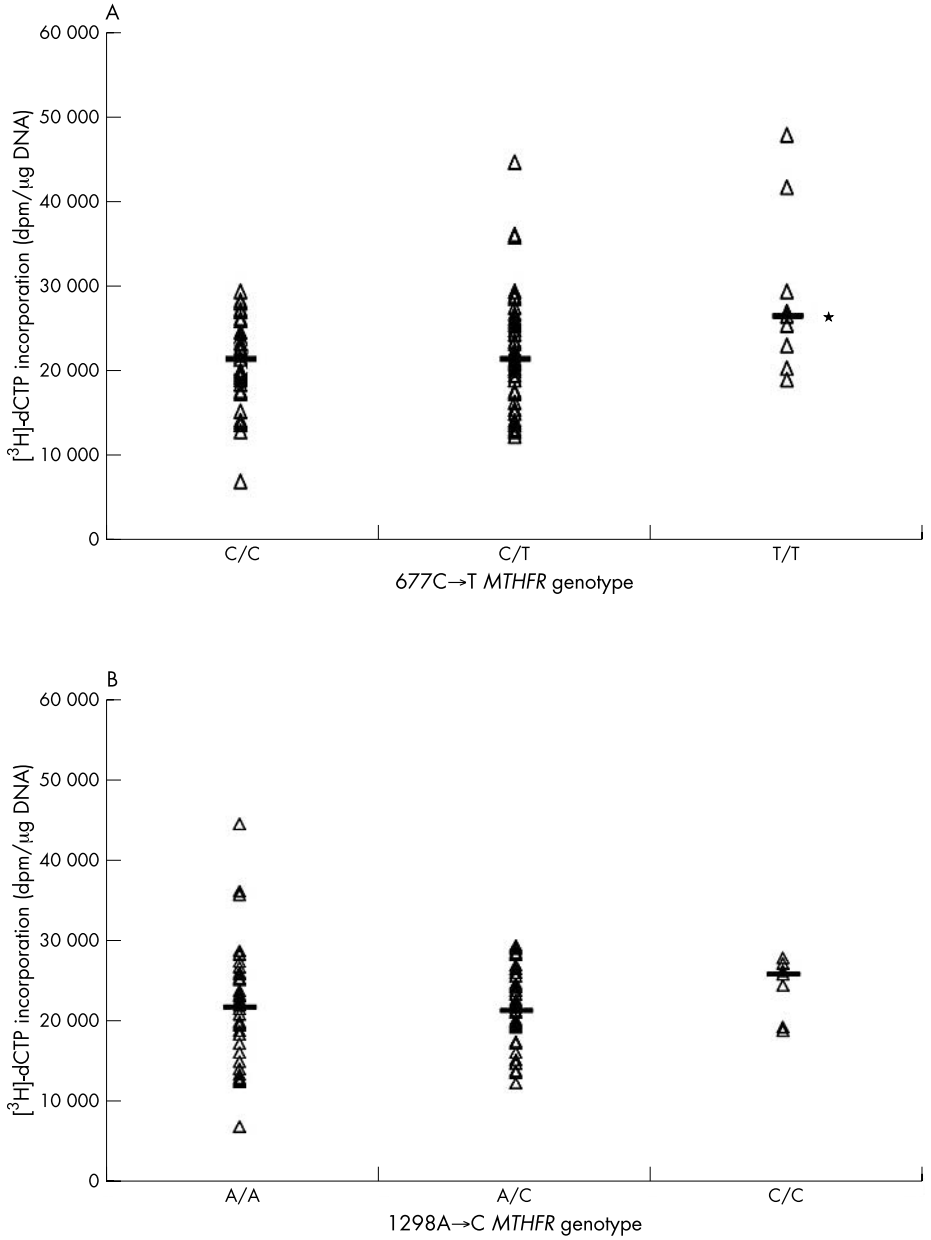

Figure 1 [^{3}H]-dCTP incorporation (dpm/μg DNA), according to (A) *MTHFR* 677C→T or (B) *MTHFR* 1298A→C genotypes, in the studied population. A Δ represents the mean of two independent determinations for each studied individual; a bar represents the median value for each genotype; *p<0.05, statistical difference (Student's *t* test) between 677TT and 677CC genotypes.

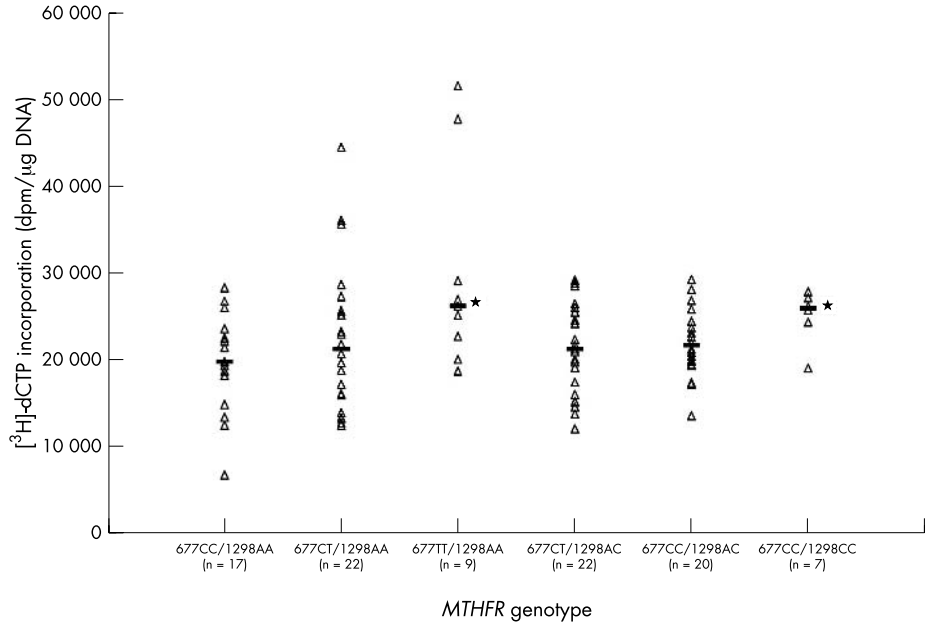

Figure 2 [^{3}H]-dCTP incorporation (dpm/µg DNA), according to *MTHFR* 677C→T and 1298A→C genotypes, in the studied population. A Δ represents the mean of two independent determinations for each studied individual; A bar represents the median value for each genotype; *$p<0.05$, statistical difference (Student's *t* test) between the 677TT/1298AA or 677CC/1298CC, and the 677CC/1298AA or 677CC/1298AA genotypes, respectively.

In conclusion, both *MTHFR* mutations, when in the homozygous state, affected DNA methylation status, although the effects were slightly less pronounced for the 1298A→C transversion. We confirmed the previous observations made by Friso et al that the 677TT *MTHFR* genotype[3] was associated with DNA hypomethylation, and we further extended this observation to other common mutation in the *MTHFR* gene, the 1298A→C transversion.

There is a growing body of evidence highlighting the role that aberrant DNA methylation plays on promotion of disease.[25–33] It is well established that abnormal DNA methylation patterns are associated with carcinogenesis.[27 30–33] Recent observations have suggested a similar pathogenic mechanism for the occurrence of vascular disease.[25–28] Accordingly, several pieces of evidence have suggested that MTHFR play a role in both cancer and vascular disease.[26 29 31–33]

Our data suggest that the 1298CC *MTHFR* genotype, independent of folate availability, and the 677TT *MTHFR* genotype with concomitant folate inadequate levels, might be potential risk factors of disease states associated with DNA hypomethylation status. Therefore, special attention should be devoted to their possible impact on disease promotion. Further research is required to evaluate the association of these two genetics variants with epigenetic alterations inducing pathology.

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