Short communication

The effect of 677C → T and 1298A → C mutations on plasma homocysteine and 5,10-methylenetetrahydrofolate reductase activity in healthy subjects

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We have studied the effect of common mutations (677C → T and 1298A → C) of the methylenetetrahydrofolate reductase (MTHFR) gene in sixty-six healthy French subjects, aged 27–47 years. Serum folate, vitamin B_{12}, and plasma total homocysteine were measured as well as the specific activity of MTHFR in lymphocytes. The frequency of subjects homozygous for the 677TT genotype was 18 %, and that of those homozygous for the 1298CC genotype was 12·5 %. The frequency of individuals heterozygous for both mutations was 23·5 %. The 1298A → C mutation was associated with decreased MTHFR specific activity in subjects with both 677CC and 677CT genotypes. This activity was 60 % for the 677CC/1298AC genotype and 52 % for the 677CC/1298CC genotype when compared with the MTHFR specific activity of the 677CC/1298AA genotype. Heterozygotes for both mutations (677CT/1298AC genotype) had 36 % of the reference specific activity. Although homocysteine levels in 677TT and 1298CC genotype subjects were higher than for other genotypes, no significant differences were observed among different genotypes. This may be due to high serum folate level in our samples, and suggests that folate therapy may be useful to prevent hyperhomocysteinaemia in homozygous mutant subjects.

Folate: Homocysteine: Methylenetetrahydrofolate reductase: Polymorphism

Increased plasma homocysteine concentrations may result from deficiencies of folic acid, vitamin B_{12} or vitamin B_6 and from genetic defects, mainly in 5,10-methylenetetrahydrofolate reductase (MTHFR) and cystathionine β-synthase (Selhub, 1999). MTHFR catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the major circulating form of folate, and a C donor for the vitamin B_{12}-dependent remethylation of homocysteine to methionine. Thermolability of MTHFR is caused by a missense mutation in exon 4 of the MTHFR gene, a cytosine (C) to thymine (T) substitution at nucleotide (677C → T), which converts an alanine (A) codon to a valine (V) codon (Frosst *et al*. 1995). The 677C → T mutation is common in different populations, with reported homozygote frequencies of 5–28 % (Schneider *et al*. 1998). A high proportion of people with the 677C → T homozygous genotype show a satisfactory homocysteine lowering response to modest daily folate supplements in the range 100–200 μg/d (Guttormsen *et al*. 1996).

Recently, a second genetic polymorphism in MTHFR, an adenine (A) to cytosine (C) substitution at position 1298 (in exon 7) has been reported (Viel *et al*. 1997; van der Put *et al*. 1998; Weisberg *et al*. 1998). This polymorphism results in the conversion of glutamic acid (E) codon to an alanine (A) codon. The homozygous state for this polymorphism has been observed in approximately 10 % of Dutch subjects (van der Put *et al*. 1998) and Canadian subjects (Weisberg *et al*. 1998). We have studied the effect of these polymorphisms on enzyme activity and fasting plasma total homocysteine (tHcy) levels in sixty-six healthy French subjects.

Abbreviations: MTHFR, 5,10-methylenetetrahydrofolate reductase; tHcy, total homocysteine.

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Subjects, materials and methods

The protocol was approved by the ethics committee of CHU Nancy, France and subjects gave their informed consent. Fasting blood was collected from sixty-six subjects (thirty males, thirty-six females) aged 27–47 years, and placed on ice immediately. All samples were processed within 2 h by centrifugation for 20 min at 2000 ***g*** and portions were stored at −70° until analysis. Lymphocytes were isolated from heparinized blood samples at room temperature by centrifugation through a Ficoll gradient with Ficoll-Hypack solution (Pharmacia, Uppsala, Sweden). Plasma tHcy and the total amount of protein- and non-protein-bound homocysteine were determined by HPLC according to Araki & Sako (1989). Serum folate and vitamin B_{12} were determined using a kit from Ciba-Corning (Medfield, MA, USA). Lymphocytes for MTHFR measurements were available for forty-two subjects (nineteen females and twenty-three males). The enzyme activities in isolated lymphocytes were measured by a radiochemical assay (Engbersen *et al.* 1995). The specific activity was measured at 37°, and activities expressed as nmol formaldehyde/mg protein per h. Protein concentrations were determined using the Bradford dye-binding procedure with a kit from Bio-Rad (Hercule, CA, USA). Genomic DNA was isolated from peripheral blood leucocytes using a Qiagen kit (Chatsworth, CA, USA) according to the manufacturer's instructions. As the 677C→T mutation creates a *Hinf* I restriction site (Frosst *et al.* 1995), and 1298A→C abolishes a *Mbo* II restriction site (van der Put *et al.* 1998), mutations were investigated by polymerase chain reaction of genomic DNA and digestion with the specific restriction enzyme. The polymerase chain reaction for the 1298A→C was carried out according to van der Put *et al.* (1998) in a total volume of 50 μl, containing 1 μM of the forward primer 5′-CTT TGG GGA GCT GAA GGA CTA CTA C and 1 μM of the reverse primer 5′-CAC TTT GTG ACC ATT CCG GTT TG, 200 μM each dNTP, 1·5 Unit Taq polymerase (Appligène-Oncor, Illkirsh, France) and 1×C of the Taq buffer. The polymerase chain reaction program on GeneAmp (Perkin Elmer, Foster, CA, USA) consisted of an initial denaturation step of 2 min at 94°, followed by thirty-five cycles of 94° (60 s), 51° (60 s) and 72° (30 s), and a final extension of 72° for 7 min. The polymerase chain reaction for the 677C→T was carried out using the forward primer 5′-TGA AGG AGA AGG TGT CTG CG and the reverse primer 5′-AGG ACG GTG CGG TGA GAG TG.

Statistical analyses were performed using StatView-5 (SAS Ins. Inc, Cary, NC, USA) on a MacIntosh computer. Results are expressed as the mean value and standard deviation. Comparisons were analysed by ANOVA followed by post-ANOVA Bonferroni/Dunn test. The distributions of serum vitamin B_{12} and folate were skewed and logarithmically transformed.

Results

The mean fasting plasma tHcy for all samples was 9·3 (SD 3·3) μmol/l. The mean serum folate and vitamin B_{12} concentrations were 15·6 (SD 8·7) (normal range 6–30) nmol/l and 359·9 (SD 158·9) (normal range 156–674) pmol/l respectively. The mean MTHFR activity was 16·7 (SD 8·2) (range 4·8–41·0) nmol formaldehyde/mg protein per h.

Subjects were divided into three groups based on either their 677C→T or their 1298A→C genotype without taking into account their status at the second polymorphic site (Table 1). In this present sample, 18 % of the subjects were homozygous for the 677T allele and 12·5 % were homozygous for the 1298C allele. Even when the status at the 1298 position was not taken into account, MTHFR specific activity in homozygous 677TT genotype individuals was 32 % and MTHFR specific activity in 677CT heterozygotes was 64 % of that seen in 677CC subjects. MTHFR specific activity in 1298CC subjects was not significantly different from that seen in 1298AA subjects or in 1298AC subjects when the allele status at position 677 was not taken into account.

When the sample was analysed to look at the effect of the two mutations in combination, 23·5 % of the subjects were 677CT/1298AC (Table 2). The 677CC/1298AC MTHFR activity was 60 % and the 677CC/1298CC MTHFR activity was 52 % of the 677CC/1298AA MTHFR activity used as a reference. Heterozygotes for both mutations (677CT/1298AC genotype) had 36 % of the reference activity. Results in Table 2 show that as has been proposed previously, the 1298A→C mutation is associated with decreased MTHFR activity (van der Put *et al.* 1998; Weisberg *et al.* 1998). The reduced MTHFR activity due to the 1298A→C mutation is less than that due to the 677C→T mutation and can be

Table 1. Plasma homocysteine, serum folate and B_{12} and methylenetetrahydrofolate reductase (MTHFR) specific activity according to genotype (Mean values and standard deviations)

Genotype …	677C→T mutation						1298A→C mutation					
	677CC		677CT		677TT		1298AA		1298AC		1298CC	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
n	29	44·5 %	25	37·5 %	12	18 %	31	46·5 %	27	41 %	8	12·5 %
Plasma homocysteine (μmol/l)*	9·0	2·7	9·3	3·3	10·7	5·3	9·4	3·7	9·0	3·3	10·8	3·1
Serum folate (nmol/l)*	16·6	9·8	13·3	5·8	15·5	7·9	14·9	7·3	14·9	8·3	17·1	11·2
Serum B_{12} (pmol/l)*	349·8	113·2	370·8	154·2	299·4	88·2	345·3	162·2	347·4	101·9	367·3	77·0
MTHFR specific activity (nmol formaldehyde/mg protein per h)	22·5[a]	8·2	14·5	4·8[b]	7·3	1·4[c]	17·3	11·1	15·8	5·2	17	2·7

[a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
* Plasma homocysteine, serum folate and B_{12} were not significantly different according to genotype (post-ANOVA Bonferroni/Dunn test).

Table 2. Effect of the 1298A → C mutation on plasma homocysteine, serum folate and B_{12} and methylenetetrahydrofolate reductase (MTHFR) specific activity according to 677C → T genotype (Mean values and standard deviations)

Genotype …	1298AA						1298AC						1298CC					
	677CC		677CT		677TT		677CC		677CT		677TT		677CC		677CT		677TT	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
n	9	14·4 %	10	14·5 %	12	18 %	12	18 %	15	23 %	–	–	8	12·5 %	–	–	–	–
Plasma homocysteine (μmol/l)*	8·4	1·9	9·0	3·1	10·7	5·3	8·2	2·8	9·7	3·6	–	–	10·8	3·1	–	–	–	–
Serum folate (nmol/l)*	16·3	8·8	13·0	5·7	16·1	7·8	16·4	10·5	13·6	6·3	–	–	17·1	11·2	–	–	–	–
Serum B_{12} (pmol/l)*	327·5	132·8	414·7	234·7	302·5	83·7	355·6	125·7	341·0	84·3	–	–	367·3	77·0	–	–	–	–
MTHFR specific activity (nmol formaldehyde/mg protein per h)	33·0[a]	8·0	16·7[b]	5·9	7·3[d]	1·3	19·7[b]	4·4	11·8[c]	1·9	–	–	17·0[b]	2·7	–	–	–	–

[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$).
* Plasma homocysteine, serum folate and B_{12} were not significantly different according to genotype (post-ANOVA Bonferroni/Dunn test).

masked when the status of subjects at position 677 is not taken into account (Table 1). A third variant in MTHFR (1397T → C) has been identified in exon 7 which can affect the detection of the 1298A → C polymorphism by *Mbo* II (Weisberg *et al*. 1998). As it was common only in a small sample of African individuals (frequency 39 % of alleles in African individuals *v*. 5 in Canadian individuals), it should not have a major effect on our results.

Discussion

It is now established that the 677C → T mutation results in a thermolabile MTHFR variant with reduced activity, but the exact physiological consequences of MTHFR thermolability are not yet known. As the alanine residue is involved in the folate-dependent stabilization of MTHFR (Frosst *et al*. 1995), replacement of the alanine by valine may alter the interaction between MTHFR and folate, leading to perturbation in conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the major circulatory form of folate.

Subjects who are homozygous for 677C → T are reported to have high plasma tHcy levels in the presence of folate insufficiency (Jacques *et al*. 1996). In this study, plasma tHcy levels were not significantly increased by either the 677C → T nor by the 1298A → C mutation. However, there was a trend to higher tHcy levels in the homozygote 677TT or 1298CC individuals. Only two of the sixty-six subjects in our study had clearly elevated plasma tHcy levels. These two subjects were men with the 677TT genotype, without the 1298A → C mutation. The first had a serum folate concentration of 5·5 nmol/l, a serum vitamin B_{12} of 307 pmol/l, and a plasma homocysteine of 18·1 μmol/l. The second had a serum folate of 9 nmol/l, a serum vitamin B_{12} of 194 pmol/l, and a plasma homocysteine of 19·1 μmol/l. The lack of a significant difference in tHcy levels may be explained by the high serum folate level in most of the homozygous 677TT individuals, and suggests that folate therapy may be useful to prevent hyperhomocysteinaemia in homozygous mutant subjects. For the novel variant, as suggested by Weisberg *et al*. (1998), the 1298A → C polymorphism could affect enzyme regulation, possibly by *S*-adenosylmethionine, an allosteric inhibitor of MTHFR, which is known to bind in the C-terminal region.

The allele frequency of the 677T allele in this present group was 37 % and the frequency of 677TT homozygotes was 18 %. In previous studies from France, homozygote frequencies of 10–18·5 % have been quoted for control subjects (Chadefaux-Vekemans *et al*. 1996; Faure-Delanef *et al*. 1997; Mornet *et al*. 1997). The allele frequency of the 1298C allele was 33 % and the frequency of 1298CC homozygotes was 12·5 %. This study in French subjects is a confirmation that the second mutation 1298A → C is a genetic polymorphism. It is interesting that to date, there has been only one reported instance where an individual has been 677TT/1298AC (Weisberg *et al*. 1998). The present study confirms that the two mutant phenotypes rarely occur together in *cis* (van der Put *et al*. 1998; Weisberg *et al*. 1998).

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References

Araki A & Sako Y (1989) Determination of free and total homocysteine human plasma by HPLC with fluorescence detection. *Journal of Chromatography* **422**, 43–52.

Chadefaux-Vekemans B, Kara MA & Thuillier L (1996) Does the prevalent mutation 677C → T in the methylenetetrahydrofolate reductase gene account for hyperhomocysteine related to cardiovascular disease. *Journal of Inherited Metabolism Disease* **19**, Suppl., 25.

Engbersen AMT, Franken DG, Boers GH, Stevens EMB, Trijbels FJM & Blom HJ (1995) Thermolabile 5,10-methylenetetrahydrofolate reductase as cause of mild hyperhomocysteinemia. *American Journal of Human Genetics* **56**, 142–150.

Faure-Delanef L, Quere I, Chasse JF, Guerassimenko O, Lesaulnier M, Bellet H, Zittoun J, Kamoun P & Cohen D (1997) Methylenetetrahydrofolate reductase thermolabile variant and human longevity. *American Journal of Human Genetics* **60**, 999–1001.

Frosst F, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJA, den Heijer M, Kluijtmans LAJ, van den Heuvel LP & Rozen R (1995) A candidate genetic risk factor for vascular disease: a common mutation MTHFR. *Nature Genetics* **10**, 111–113.

Guttormsen AB, Ueland PM, Nesthus I, Nygard O, Schneede J, Vollset SE & Refsum H (1996) Determinants and vitamin responsiveness of intermediate hyperhomocysteinemia (⩾ 40 μmol/liter). The Hordaland homocysteine study. *Journal of Clinical Investigation* **98**, 2174–2183.

Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, Selhub J & Rozen R (1996) Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation* **93**, 7–9.

Mornet E, Muller F, Lenvolsé A, Delezoide A-L, Col J-Y, Simon-Bouy B & Serre J-L (1997) Screening of the C677T mutation on the methylenetetrahydrofolate gene in French patients with neural tube defects. *Human Genetics* **100**, 512–514.

Schneider JA, Rees DC, Liu YT & Clegg JB (1998) Worldwide distribution of a common methylenetetrahydrofolate reductase mutation. *American Journal of Human Genetics* **62**, 1258–1260.

Selhub J (1999) Homocysteine metabolism. *Annual Review of Nutrition* **19**, 217–246.

van der Put NMJ, Gabreëls F, Stevens MB, Smeitink JAM, Trijbels FJM, Eskes TKAB, van der Heuvel LP & Blom HJ (1998) A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects? *American Journal of Human Genetics* **62**, 1044–1051.

Viel A, DallAgnese L, Simone F, Canzonieri V, Capozzi E, Visentin MC, Valle R & Boiocchi M (1997) Loss of heterozygosity at the 5,10-methylenetetrahydrofolate reductase locus in human ovarian carcinomas. *British Journal of Cancer* **75**, 1105–1110.

Weisberg I, Tran P, Christensen B, Sibani S & Rozen R (1998) A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Molecular Genetics and Metabolism* **64**, 169–172.