MOLECULAR GENETICS AND METABOLISM **64,** 169–172 (1998)
ARTICLE NO. GM982714

A Second Genetic Polymorphism in Methylenetetrahydrofolate Reductase (MTHFR) Associated with Decreased Enzyme Activity

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Received April 9, 1998

A common mutation in methylenetetrahydrofolate reductase (MTHFR), C677T, results in a thermolabile variant with reduced activity. Homozygous mutant individuals (approximately 10% of North Americans) are predisposed to mild hyperhomocysteinemia, when their folate status is low. This genetic–nutrient interactive effect is believed to increase the risk for neural tube defects and vascular disease. In this communication, we characterize a second common variant in MTHFR (A1298C), an E to A substitution. Homozygosity was observed in approximately 10% of Canadian individuals. This polymorphism was associated with decreased enzyme activity; homozygotes had approximately 60% of control activity in lymphocytes. Heterozygotes for both the C677T and the A1298C mutation, approximately 15% of individuals, had 50–60% of control activity, a value that was lower than that seen in single heterozygotes for the C677T variant. No individuals were homozygous for both mutations. Additional studies of the A1298C mutation, in the absence and presence of the C677T mutation, are warranted, to adequately address the role of this new genetic variant in complex traits. A silent genetic variant, T1317C, was identified in the same exon. It was relatively infrequent (allele frequency 5%) in our study group, but was quite common in a small sample of African individuals (allele frequency 39%).

Key Words: **methylenetetrahydrofolate reductase; homocysteine; folic acid; polymorphism.**

Methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) synthesizes 5-methyltetrahydrofolate, the primary circulatory form of folate, which serves as a methyl donor for homocysteine remethylation to methionine. Fourteen rare mutations in MTHFR have been described; these are associated with severe enzymatic deficiency and homocystinuria (1). One common polymorphism (C677T) results in a thermolabile enzyme with reduced specific activity (approximately 35% of control values in homozygous mutant individuals) (2). Several studies have reported an increased risk for spina bifida in children with the homozygous mutant genotype for C677T (3,4). In addition, by virtue of the role of MTHFR in folate-dependent homocysteine metabolism, this mutation predisposes to mild hyperhomocysteinemia, a risk factor for vascular disease, in the presence of low folate status (1,2). Recently, in a study of loss of heterozygosity at the MTHFR locus in ovarian carcinomas, Viel *et al.* (5) identified a sequence change (C1298A) but did not elaborate on this particular mutation. To determine the frequency of this variant and to assess its potential impact on enzyme function, we studied this mutation in a group of NTD cases and in their mothers, as well as in their respective controls. We also identified a silent genetic variant, T1317C, in an African-American patient with homocystinuria and examined its frequency in a subset of the above study group, as well as in a small panel of DNAs from Africans.

PATIENTS AND METHODS

Patients with spina bifida and mothers of patients were recruited from the Spina Bifida Clinic at the

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1096-7192/98 $25.00

TABLE 1
Genotype Distributions, MTHFR Activity (nmol formaldehyde/mg protein/h), and Total Plasma Homocysteine (tHcy, μM) for Mothers and Children

	E/E			E/A			A/A		
	A/A	A/V	V/V	A/A	A/V	V/V	A/A	A/V	V/V
				Mothers (n = 141)					
No.	24	32	19	27	26	0	13	0	0
%	17	23	13	19	18	0	9	0	0
MTHFR	49.0 ± 18.9 (14)	33.0* ± 10.8 (19)	15.7* ± 4.5 (11)	45.0 ± 16.0 (15)	30.2* ± 19.3 (15)	—	32.1* ± 9.0 (7)	—	—
tHcy	9.5 ± 3.1 (24)	10.0 ± 3.2 (32)	12.2** ± 7.1 (19)	8.4 ± 2.1 (25)	10.0 ± 3.1 (26)	—	9.5 ± 2.0 (13)	—	—
				Children (n = 133)					
No.	23	43	18	20	15	1	13	0	0
%	17	32	13	15	11	1	10	0	0
MTHFR	52.0 ± 17.0 (12)	38.2* ± 15.0 (27)	16.2* ± 5.3 (11)	35.7* ± 9.7 (18)	26.1* ± 5.0 (9)	21.6 (1)	29.5* ± 10.3 (6)	—	—
tHcy	7.6 ± 2.5 (23)	8.2 ± 3.0 (43)	9.7** ± 5.1 (18)	7.5 ± 2.3 (20)	8.1 ± 2.8 (15)	9.5 (1)	7.4 ± 1.5 (13)	—	—

Note. The three A1298C genotypes and the three C677T genotypes are designated by the amino acid codes: EE, EA, AA, and AA, AV, VV, respectively. Statistical significance was assessed by Student's t test, in comparison with EEAA values. *($P < 0.05$); **($P \leq 0.07$). Standard deviations are given and parentheses indicate the number of individuals for whom MTHFR activities and homocysteine levels were available.

Montreal Children's Hospital following approval from the Institutional Review Board. Control children and mothers of controls were recruited from the same institution. Blood samples were used to prepare DNA from peripheral leukocytes, to assay MTHFR activity in lymphocyte extracts, and to measure total plasma homocysteine (tHcy). Details of these methodologies are described elsewhere (6,7). The presence of the C677T mutation (A to V) was evaluated by PCR and *Hin*fI digestion, as reported (2). The A1298C mutation was initially examined by PCR and *Mbo*II digestion, as described in the original report (5). The silent mutation, T1317C, was identified by SSCP and sequence analysis in a patient with severe MTHFR deficiency and homocystinuria. This patient, an African-American female, already carries a previously described splice mutation (patient 354 (8)). Since this mutation also creates a *Mbo*II site and results in a digestion pattern identical to that of the A1298C mutation, distinct artificially created restriction sites were used to distinguish between these two mutations. Detection of the A1298C polymorphism was performed with the use of the sense primer 5′-GGGAGGAGCTGACCAGTGCAG-3′ and the antisense primer (5′-GGGGTCAGGCCAGGGGCAG-3′), such that the 138-bp PCR fragment was digested into 119- and 19-bp fragments by *Fnu*4HI in the presence of the C allele. An antisense primer (5′-GGTTCTCCCGAGAGGTAAAGATC-3′), which introduces a *Taq*I site, was similarly designed to identify the C allele of the T1317C polymorphism. Together with a sense primer (5′-CTGGGGATGTGGTGGCACTGC-3′), the 227-bp fragment is digested into 202- and 25-bp fragments.

RESULTS

The frequencies of the three genotypes for the A1298C mutation (EE, EA, and AA) were not different between case and control mothers, nor between case and control children (data not shown). Consequently, all the mothers and all the children were grouped together for analyses (Table 1). Nine percent of mothers had the homozygous AA genotype while 37% were heterozygous. This frequency is quite similar to the frequency of the homozygous mutant genotype (VV) for the C677T polymorphism (2). In our report of the MTHFR human cDNA sequence, the cDNA contained the C nucleotide at bp 1298. Therefore, the report by Viel *et al.* (5) referred to this change as a C1298A substitution. However, since the A nucleotide is clearly the more frequent base at this position, the A1298C nomenclature was chosen for our report.

Since the C677T mutation (A to V) is known to decrease MTHFR activity and increase homocysteine levels, the three genotype groups for the new A1298C (E to A) mutation were further stratified by the genotype for the A to V mutation, to avoid the confounding influence of the latter polymorphism on MTHFR activity and homocysteine levels. The fre-

quencies of the nine genotypes, with MTHFR activity and homocysteine levels for each genotype, are shown in Table 1. If the mothers and children without either mutation, i.e., EE/AA are used as the reference (control) group, the mothers and children that are homozygous for the A1298C change (AAAA) have approximately 65 and 57%, respectively, of control MTHFR activity. Heterozygotes for the C677T change alone (EEAV) have approximately 70% of control activity, as reported in other studies, while double heterozygotes (EAAV), 18% of mothers and 11% of children, have an additional loss of activity (approximately 62 and 50% of control values, respectively).

Homocysteine levels were not significantly increased by the A1298C mutation, but homocysteine was elevated (with borderline significance, $P \leq 0.07$) in mothers and children who were homozygous for the C677T change, as previously reported (2). The small number of individuals who were homozygous for the A1298C mutation ($n = 13$) may have influenced the power of our statistical analyses and precluded an investigation of the genetic–nutrient interactive effect that leads to mild hyperhomocysteinemia, as seen in individuals with the C677T mutation (1).

The T1317C substitution does not alter the amino acid (phenylalanine) and is likely a benign change, although a splicing defect cannot be ruled out at the present time. In an evaluation of 38 control mothers from this study, 2 were found to be heterozygous and 1 was identified as a homozygote, resulting in an allele frequency of 5% (4/76). Since this substitution was identified in an African-American female, control African individuals were also examined ($n = 9$). Seven of these were heterozygous, resulting in an allele frequency of 39% (7/18).

DISCUSSION

The A1298C mutation clearly reduces MTHFR activity, albeit to a lesser extent than the C677T mutation. Consequently its effect on homocysteine levels is also attenuated and, in fact, may only be significant when an individual carries both mutations and/or has poor nutrient status. However, since double heterozygotes are estimated to represent approximately 15% of the population, this variant should be examined in conjunction with the C677T variant in studies of hyperhomocysteinemia.

The A1298C mutation is clearly polymorphic in Canadian individuals and should be examined in other populations. The A nucleotide is likely to be the ancestral sequence since it represents the more common allele, although the original human MTHFR cDNA sequence (GenBank Accession Number U09806) carried the C nucleotide. This polymorphism is similar in frequency to the C677T polymorphism. Presumably the two substitutions arose separately on a A1298/C677 or E/A haplotype, since the haplotype with both substitutions (C1298/T677 or A/V) is extremely rare. One such haplotype was seen in a child with the EAVV genotype, suggesting a recombinant chromosome.

Doubly homozygous individuals (AAVV) were not observed in this study. Since the double mutation in cis is rare, it is possible that we simply did not study enough alleles. Larger studies in other populations might result in the identification of these individuals. Presumably the MTHFR activity would be even lower and homocysteine levels might be higher than those observed thus far.

The C677T polymorphism in exon 4 is within the N-terminal catalytic domain of the enzyme whereas the A1298C polymorphism in exon 7 is within the C-terminal regulatory domain. The more dramatic effect on enzyme activity with the first polymorphism may be a consequence of its location within the catalytic region. The second polymorphism could affect enzyme regulation, possibly by *S*-adenosylmethionine, an allosteric inhibitor of MTHFR, which is known to bind in the C-terminal region (1).

Many studies have examined the effects of the C677T polymorphism on MTHFR enzyme activity and on homocysteine levels. Although the correlation between the presence of this substitution and decreased enzyme activity/increased homocysteine levels has been quite good, the variability in results, particularly in heterozygous individuals, may reflect the presence of a second common variant in the population.

The third variant, T1317C, was present on 5% of alleles in Canadian individuals but appears to be extremely common in individuals of African ancestry. The methodology outlined in this report should be used to assess the frequency of the A1298C and T1317C in other populations, since the use of the *Mbo*II restriction site for analysis of the A1298C change, as first reported (5), would not discriminate between the two polymorphisms.

The C677T mutation is a risk factor for hyperhomocysteinemia and has been implicated in both neural tube defects and vascular disease. The identification of a second variant (A1298C), with clearly

reduced enzyme activity and potential impact on homocysteine levels, warrants additional investigation of this new polymorphism in these multifactorial disorders.

ACKNOWLEDGMENTS

We thank Drs. Laura Arbour, Brian Gilfix, David Rosenblatt, and Patricia Forbes for their participation in the original study from the Spina Bifida Clinic, Montreal Children's Hospital (Christensen *et al.*, submitted for publication). This work was supported by the Medical Research Council of Canada.

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