Q J Med 1997; **90**:593–596

Elevated plasma total homocysteine and C677T mutation of the methylenetetrahydrofolate reductase gene in patients with spina bifida

A.L. BJØRKE-MONSEN, P.M. UELAND, J. SCHNEEDE, S.E. VOLLSET[1] and H. REFSUM

From the Department of Pharmacology and [1]Section of Medical Informatics and Statistics, University of Bergen, Norway

Received 19 June 1997

Summary

Total plasma homocysteine (tHcy) was significantly higher in 28 children with spina bifida (median 6.05 μmol/l) as compared with 76 controls (median 4.94 μmol/l). This difference was confined to a subgroup of patients (16/28) with one or two C677T-mutated alleles in the methylenetetrahydrofolate reductase gene. Since we found no significant difference between patients and controls in serum folate, erythrocyte folate, serum cobalamin or serum methylmalonic acid, which were within the normal range for both patients and controls, the elevated tHcy could not be attributed to vitamin deficiencies. Our findings point to an additional genetic defect involving folate-dependent enzymes in a subgroup of patients with neural-tube defects.

Introduction

Both observational and intervention studies have demonstrated that periconceptional administration of folate reduces the occurrence and the recurrence rate of neural-tube defects (NTD).[1,2] Women carrying a NTD foetus do not usually have overt folate deficiency, but recent data suggest impaired folate and possibly vitamin B12 homeostasis,[3] which is in accordance with elevated plasma total homocysteine (tHcy) in these women.[4,5]

The C677T mutation in the gene coding for the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) causes a thermolabile enzyme variant with low enzyme activity and thereby impaired formation of 5-methyltetrahydrofolate, which is involved in the remethylation of Hcy to methionine.[6–8] The C677T mutation has been associated with elevated tHcy, especially under conditions of low folate status.[9–11]

Increased prevalence of the C677T mutation in the MTHFR gene has been demonstrated in children with neural-tube defects and in their parents,[12–14] but the relevance of the C677T mutation for the development of NTD has recently been questioned by some authors.[15–18]

In this preliminary study, we compared the tHcy level in children with spina bifida with that in control children. tHcy level was higher in spina bifida, but this was confined to individuals with one or two C677T mutated alleles.

Methods

The patient group consisted of 28 children with spina bifida aged 5 days to 16 years, median age 5.3 years. They were recruited at the paediatric clinic during routine examination ($n=26$), or immediately following birth ($n=2$). The control group included 76 children aged 5 days to 14.5 years, median age 5.6 years. The controls were recruited among healthy newborns ($n=12$) and patients admitted to a paediatric outpatient clinic for minor diseases ($n=64$). None

Address correspondence to Dr A.L. Bjørke-Monsen, Department of Pharmacology, University of Bergen, Armauer Hansens hus, 5021 Bergen, Norway

of the controls had birth defects, and they were not using any medications. The protocol was approved by the local ethics committee, and written informed consent was obtained from parents of both patients and controls.

The C677T mutation in the MTHFR gene was determined in all 28 patients and in 68 of the controls by PCR amplification of DNA followed by restriction enzyme digestion with *Hinf*I. The analysis of DNA fragments was performed with capillary electrophoresis.[19]

Serum and erythrocyte folate were assayed using the Quantaphase folate radioassay (Bio Rad). Serum cobalamin was determined with a microparticle enzyme intrinsic factor assay run on an IMX system (Abbott). tHcy in EDTA plasma was determined by an automated method based on HPLC and fluorescence detection.[20] Serum methylmalonic acid (MMA) was assayed by a method based on capillary electrophoresis and laser-induced fluorescence detection.[21]

Conditional logistic regression was used to estimate the relative risk (odds ratio, OR) of neural-tube defects for various levels of tHcy with adjustment for age (by stratification in age groups 0–2, 2–5, 6–9,10–16 years), sex, serum creatinine, serum cobalamin and serum folate. Regression analyses were performed separately for individuals without and with one or two alleles of the C677T MTHFR mutation. A test-of-effect modification of the tHcy-NTD relation by MTHFR status was performed with a product term of a linear tHcy (three levels) and binary MTHFR representation with both main effects present in the model. Analyses were carried out with LogXact Turbo version 1.1. (Cytel Software).

Results

Of the control children, 4/68 (5.9%) were homozygous for the C677T mutation (TT genotype), 22/68 (32.3%) were heterozygous (CT genotype) and 42/68 (61.8%) were without the mutation (CC genotype). Among the NTD children, the distribution between the TT, CT and CC genotypes were 1/28 (3.6%), 15/28 (53.6%) and 12/28 (42.9%), respectively.

Due to the low number of homozygous subjects, the TT and CT genotypes were combined into one group.

There were no significant differences in levels of serum creatinine, serum and erythrocyte folate, serum cobalamin or serum MMA between patients and controls. Nor did these parameters differ between the CC group vs. the CT/TT group in either patients or controls (Table 1).

Plasma tHcy was significantly higher in the NTD patients than in controls (median: 6.05 μmol/l vs. 4.94 μmol/l, $p<0.0001$). Notably, this difference was confined to the subgroup of patients with one or two mutated alleles (the combined CT/TT group). Within this group, tHcy was markedly higher in patients (median 6.86 μmol/l) than in controls (4.84 μmol/l) (Table 1).

With adjustment for age and sex (Model A) there was a strong and highly significant association of tHcy to NTD (Table 2). The ORs for NTD comparing intermediate (5–6.99 μmol/l) and high ($\geq$7.0 μmol/l) tHcy levels to the reference tHcy level ($<$5.0 μmol/l) were 2.5 and 11.0, respectively (p for trend = 0.001). There was no association among individuals without the C677T mutation, whereas tHcy ORs were 5.3 and 43.6 (p for trend = 0.004) in the subgroup with one or two T alleles. The tHcy-NTD association was significantly stronger in the CT/TT compared to the CC group, (p for effect modification = 0.01). Additional adjustment for serum folate, serum cobalamin and serum creatinine (Model B) weakened the overall correlation of tHcy with NTD slightly, but the effect modification by MTHFR status and the strong correlation between tHcy and CT/TT individuals remained.

Discussion

This study addresses the possibility of impaired homocysteine metabolism in children with NTD. Despite the small study group (n=28), we found a markedly elevated plasma tHcy in NTD patients compared to controls. Notably, the elevation was confined to patients with one or two mutated alleles (CT/TT genotypes), whereas tHcy was equal in patients and controls without the mutation.

We observed small, non-significant differences in serum and erythrocyte folate and in serum cobalamin between patients and controls and between genotypes. The vitamin levels were within the normal range for both patients and controls (Table 1). These findings do not suggest that elevated tHcy is related to vitamin deficiency. A possible explanation is impaired cellular metabolism of folate or cobalamin, which in part is conferred by the C677T mutation in MTHFR. However, in adults at least, homozygosity and heterozygosity in particular, are not associated with hyperhomocysteinaemia in subjects with normal serum folate.[11] This points to the possibility of an additional genetic defect in cellular folate or cobalamin function in NTD subjects, and methionine synthase is a possible candidate locus.[5,14]

The present finding should be confirmed in larger studies which should include search for compound genetic defects involving folate-dependent enzymes.

Acknowledgements

This work was supported by grants from the Norwegian Research Council. The authors thank Dr

Table 1 Vitamins, MMA and Hcy in relation to MTHFR status

	All children	MTHFR genotype		p^*
		CC	CT/TT	
Controls	(*n*=76)	(*n*=42)	(*n*=22/4)	
Patients	(*n*=28)	(*n*=12)	(*n*=15/1)	
Red-cell folate (nmol/l)				
Controls	400 (339–524)	402 (341–530)	395 (338–497)	0.8
Patients	401 (318–501)	440 (363–517)	358 (305–497)	0.9
p†	0.4	0.8	0.6	
Serum folate (nmol/l)				
Controls	12.2 (9.1–17.5)	13.1 (9.1–18.9)	11.2 (9.3–15.5)	0.4
Patients	10.8 (6.6–18.5)	12.1 (10.3–19.1)	9.7 (6.5–16.6)	0.5
p†	0.2	0.6	0.4	
Serum cobalamin (pmol/l)				
Controls	609 (370–711)	609 (379–700)	526 (324–720)	0.8
Patients	507 (426–761)	505 (426–823)	507 (396–694)	0.6
p†	0.7	0.6	0.8	
Serum MMA (μmol/l)				
Controls	0.12 (0.08–0.17)	0.11 (0.07–0.18)	0.12 (0.08–0.17)	0.4
Patients	0.12 (0.05–0.17)	0.15 (0.10–0.17)	0.12 (0.05–0.18)	0.5
p†	0.8	0.6	0.3	
Plasma tHcy (μmol/l)				
Controls	4.94 (4.17–6.11)	5.14 (4.15–6.26)	4.84 (4.21–6.02)	0.9
Patients	6.05 (4.83–8.20)	5.14 (4.54–6.05)	6.86 (5.91–10.52)	<0.01
p†	<0.0001	0.6	<0.0001	

Data are medians (interquartile range). MTHFR, methylenetetrahydrofolate reductase; CC, normal genotype without the C677T mutation; CT, heterozygous genotype; TT, homozygous genotype. Unpaired t-tests: p^*, CC vs. CT/TT genotypes; *p*†, controls vs. patients.

Table 2 Odds ratios for NTD by levels of plasma tHcy overall and by MTHFR status

	NTD (*n*)	Controls (*n*)	Model A OR (95% CI)	Model B OR (95% CI)
All children	28	76		
tHcy (μmol/l)				
<5.00	8	38	1	1
5–6.99	11	32	2.5 (0.81–7.6)	1.9 (0.49–7.1)
⩾7.00	9	6	11.0 (2.4–49.8)	6.5 (0.99–42.6)
p for trend			0.001	0.06
CC genotypes	12	42		
tHcy (μmol/l)				
<5.00	6	17	1	1
5–6.99	5	21	1.5 (0.29–7.8)	2.7* (0.11–66.8)
⩾7.00	1	4	1.3 (0.10–16.2)	
p for trend			0.73	0.98
CT/TT genotypes	16	26		
tHcy (μmol/l)				
<5.00	2	15	1	1
5–6.99	6	9	5.3 (0.90–31.2)	4.5 (0.61–32.1)
⩾7.00	8	2	43.6 (3.1–619)	44.4 (1.91–1033)
p for trend			0.004	0.02
p for effect modification			0.01	0.03

Model A, stratified by age (<2, 2–5, 6–9, 10–16 years) and adjusted for sex by conditional logistic regression. Model B, stratified by age and adjusted for sex, serum creatinine, serum cobalamin and serum folate. *tHcy ⩾5 μmol/l combined into one group.

A. Bakke at the Department of Urology for recruiting patients, and the staff at the Department of Pediatrics, University of Bergen, for help in collecting the blood samples.

References

1. MRC Vitamin Study Research Group. Prevention of neural tube defects—results of the Medical Research Council vitamin study. *Lancet* 1991; **338**:131–7.
2. Czeizel AE, Dudas I. Prevention of the 1st occurrence of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med* 1992; **327**:1832–5.
3. Kirke PN, Molloy AM, Daly LE, Burke H, Weir DG, Scott JM. Materal plasma folate and vitamin B12 are independent risk factors for neural tube defects. *Q J Med* 1993; **86**:703–8.
4. Steegers-Theunissen RPM, Boers GHJ, Trijbels FJM, Eskes TKAB. Neural-tube defects and derangement of homocysteine metabolism. *N Engl J Med* 1991; **324**:199–200.
5. Mills JL, McPartlin JM, Kirke PN, Lee YJ, Conley MR, Weir DG, Scott JM. Homocysteine metabolism in pregnancies complicated by neural-tube defects. *Lancet* 1995; **345**:149–51.
6. Kang SS, Wong PWK, Zhou J, Sora J, Lessick M, Ruggie N, Grcevich G. Thermolabile methylenetetrahydrofolate reductase in patients with coronary artery disease. *Metabolism* 1988; **37**:611–13.
7. Kang SS, Zhou J, Wong PWK, Kowalisyn J, Strokosch G. Intermediate homocysteinemia: A thermolabile variant of methylenetetrahydrofolate reductase. *Am J Hum Genet* 1988; **43**:414–21.
8. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GHJ, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozen R. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nature Genet* 1995; **10**:111–13.
9. Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, Selhub J, Rozen R. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation* 1996; **93**:7–9.
10. Kluijtmans LAJ, van den Heuvel LPWJ, Boers GHJ, Frosst P, van Oost BA, den Heijer M, Stevens EMB, Trijbels JMF, Rozen R, Blom HJ. Molecular genetic analysis in mild hyperhomocysteinemia: A common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease. *Am J Hum Genet* 1996; **58**:35–41.
11. Guttormsen AB, Ueland PM, Nesthus I, Nygård O, Schneede J, Vollset SE, Refsum H. Determinants and vitamin responsiveness of intermediate hyperhomocysteinemia (>40 μmol/l). The Hordaland homocysteine study. *J Clin Invest* 1996; **98**:2174–83.
12. van der Put NMJ, Steegers-Theunissen RPM, Frosst P, Trijbels FJM, Eskes TKAB, van den Heuvel LP, Mariman ECM, den Heyer M, Rozen R, Blom HJ. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. *Lancet* 1995; **346**:1070–1.
13. Whitehead AS, Gallagher P, Mills JL, Kirke PN, Burke H, Molloy AM, Weir DG, Shields DC, Scott JM. A genetic defect in 5,10 methylenetetrahydrofolate reductase in neural tube defects. *Q J Med* 1995; **88**:763–6.
14. Ou CY, Stevenson RE, Brown VK, Schwartz CE, Allen WP, Khoury MJ, Rozen R, Oakley GPJ, Adams MJJ. 5,10 metylenetetrahydrofolate reductase genetic polymorphism as a risk factor for neural tube defects. *Am J Med Genet* 1996; **63**:610–14.
15. Posey DL, Khoury MJ, Mulinare J, Adams MJ, Ou CY. Is mutated MTHFR a risk factor for neural tube defects? *Lancet* 1996; **347**:686–7.
16. Papapetrou C, Lynch SA, Burn J, Edwards YH. Methylenetetrahydrofolate reductase and neural tube defects. *Lancet* 1996; **348**:58.
17. de Franchis R, Sebastio G, Mandato C, Andria G, Mastroiacovo P. Spina bifida, 677T→C mutation, and role of folate. *Lancet* 1995; **346**:1703.
18. Wilcken DEL, Wang XL. Relevance to spina bifida of mutated methylenetetrahydrofolate reductase. *Lancet* 1996; **347**:340.
19. Ulvik A, Refsum H, Kluijtmans LAJ, Ueland PM. The C677T mutation of the methylenetetrahydrofolate reductase gene determined in human blood or plasma by a method involving multiinjection capillary electrophoresis and laser-induced fluorescence detection. *Clin Chem* 1996; **43**:2.
20. Fiskerstrand T, Refsum H, Kvalheim G, Ueland PM. Homocysteine and other thiols in plasma and urine: Automated determination and sample stability. *Clin Chem* 1993; **39**:263–71.
21. Schneede J, Ueland PM. Application of capillary electrophoresis with laser-induced fluorescence detection for routine determination of methylmalonic acid in human serum. *Anal Chem* 1995; **34**:812–19.