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Comorbidity of 5,10-methylenetetrahydrofolate reductase and methionine synthase gene polymorphisms and risk for neural tube defects

EDITOR—Neural tube defects (NTDs) are among the most common and devastating birth defects. NTDs result from an incomplete closure of the neural tube, and include malformations of the skull, brain, meninges, spinal cord, and vertebral column. Recent evidence suggests that closure of the neural tube occurs in five separate sites which then fuse during the fourth week of gestation; NTDs occur when one site fails to close or two sites fail to fuse.[1]

During the last decade, periconceptional folic acid supplementation has been shown to reduce the risk of occurrence[2] and recurrence[3] of NTDs. Women with an NTD affected pregnancy do not usually have overt signs of folate deficiency, although decreased erythrocyte folate concentration, the index known to reflect whole body folate stores, has been reported.[4 5] In addition, it has been reported that women with NTD pregnancies have raised homocysteine concentrations in plasma and amniotic fluid,[6 7] suggesting that folate metabolism may be altered in these women.

5,10-methylenetetrahydrofolate reductase (MTHFR) catalyses the reaction from 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which serves as methyl donor for the remethylation of homocysteine to methionine.[8] A substitution (C to T) at the highly conserved nucleotide 677 of the *MTHFR* gene has been described, which results in the conversion of an alanine (Ala) to a valine (Val) residue and increased in vitro thermolability of the enzyme.[9] In vivo, the thermolabile *MTHFR* mutant is known to result in raised plasma homocysteine concentrations when folate nutriture is inadequate.[10] The frequency of homozygosity for this mutation is approximately 9% for various populations, but is higher in French Canadian, Italian, and Hispanic populations, and lower in African-American populations.[9 11–13]

The C677T mutation has been reported to be a genetic risk factor for NTDs.[11 14–17] However, the significance of this mutation as an NTD risk factor in different populations has recently been questioned. Speer *et al*[20] found no evidence of *MTHFR* C677T polymorphism as a risk factor for lumbosacral spina bifida in American white patients. Weitkamp *et al*[21] also reported that the C677T polymorphism in *MTHFR* was not associated with NTD risk in a population of mixed ethnic origins. Papapetrou *et al*[18] found no evidence for an association between the 677T allele and the incidence of NTDs in a British population. Similarly, in French, German, and American populations, the distribution of the C677T mutation was the same in fetuses with NTDs and controls.[12 19 22] Ubbink *et al*[23] reported that homozygosity for the C677T mutation does not constitute a genetic risk factor for NTDs in rural South African blacks. The low frequency of *MTHFR* C677T polymorphism in African-Americans,[13] coupled with the lower incidence of NTDs in blacks,[24] suggests that analysis of the association between *MTHFR* polymorphisms and NTD risk in a large cohort of African-Americans would provide new information relative to the importance of this association in this population group. These investigations suggest that the frequency of this mutation and its associated risk for NTD may be population dependent or dependent on folate nutriture. It is possible that the *MTHFR* mutation predisposes the fetus to the development of NTDs, but they only occur if the maternal and/or fetal folate status are suboptimal; hence, periconceptional folic acid supplementation may overcome this genetic defect. Therefore, a careful evaluation of the effect of polymorphism in association with folate nutriture is warranted.

We recently reported that C677T *MTHFR* polymorphisms and raised homocysteine levels in amniotic fluid appeared to be disproportionately associated with NTDs spanning the cervical-lumbar spine, lumbosacral spine, and occipital encephalocele.[25 26] These results suggest that periconceptional folic acid supplementation may prevent defects at these sites, but not at other sites of neural tube closure.

The gene for human methionine synthase (MS), which catalyses the reaction to form methionine from homocysteine, has recently been cloned, and a common polymorphism has also been identified.[27–29] The polymorphism is an A to G substitution at base pair (bp) 2756, converting an aspartic acid (Asp) residue into a glycine (Gly). Although MS plays an important role in homocysteine metabolism, this polymorphism has not been reported to be a risk factor for NTD formation,[30–33] and, to our knowledge, comorbidity of *MTHFR* and *MS* polymorphisms for NTDs has never been evaluated. Potential comorbidity of these enzymes may be of significance because MS and MTHFR are both key enzymes in homocysteine metabolism, and altered homocysteine metabolism has been implicated in the development of NTDs.[6 7]

We determined *MTHFR* and *MS* genotypes using DNA isolated from amniotic fluid cells of fetuses with NTDs and of those without any apparent malformations, and evaluated potential associations between polymorphisms in these two genes as a risk factor for the development of NTDs. The study was approved by the Institutional Review Board of the University of Alabama at Birmingham. The computerised genetics database at the University of Alabama Prenatal Genetics Clinic was used to identify cases in which NTDs were diagnosed by ultrasound and amniocentesis, and confirmed by neonatal examination or necropsy, between 1988 and 1997. Excess stored second trimester amniotic fluid samples from 82 women with fetuses with confirmed NTDs (cases) and from 84 women with normal pregnancies (controls) were analysed. For each case selected, we identified control samples obtained from women who had undergone amniocentesis at the same clinic but had a fetus confirmed to be normal by ultrasound, karyotype, and newborn examination. Each acceptable control was randomly assigned a computer generated priority number (SASS RANUI function) and sorted accordingly. Samples were then retrieved from storage according to their priority number; the first adequate sample located was selected as a control. Cases and controls were matched for race, maternal age, and month and year of amniocentesis. The case and control groups each had a composition of 83% white, 16% African-American, and 1% other. The mean (SD) maternal ages of cases and controls were not significantly different (26.3 (SD 5.3) and 28.1 (SD 4.9) years, respectively).

The amniotic fluid samples were collected aseptically under ultrasound guidance by an experienced operator, and were stored at −70°C until analysis. A 200 µl aliquot of amniotic fluid was centrifuged for five minutes at 13 000 × *g*. After removing 150 µl of supernatant, the remaining pellet (50 µl) was suspended in 300 µl Cell Lysis Solution and DNA was isolated (Puregene DNA Isolation Kit, Gentra Systems, Minneapolis, MN). Isolated DNA was resus-

Table 1 Odds ratios and 95% confidence intervals of 5,10-methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (MS) genotypes in fetuses with NTDs (cases) and controls

Genotype	NTDs (cases)	Controls	Odds ratio	95% confidence interval
MTHFR				
Ala/Ala	43/82 (52%)	63/76 (83%)	1.0	
Ala/Val	30/82 (37%)	11/76 (14%)	4.0	1.8–8.8
Val/Val	9/82 (11%)	2/76 (3%)	6.6	1.3–24.9
MS				
Asp/Asp	59/77 (77%)	70/84 (83%)	1.0	
Asp/Gly	18/77 (23%)	13/84 (14%)	1.6	0.7–3.6
Gly/Gly	0/77 (0%)	1/84 (1%)	—	—

pended in 25 µl Tris-EDTA buffer and stored at −20°C until analysis. The C677T mutation in the *MTHFR* gene was determined by polymerase chain reaction (PCR) amplification of DNA using an exonic and intronic primer pair that generates a 198 bp fragment.[9] Thirty eight cycles of PCR were performed for one minute at 94°C, one minute at 60°C, and two minutes at 72°C, followed by a 10 minute elongation at 72°C at the end of the cycles. The A2756G polymorphism in the *MS* gene was determined by PCR amplification using a primer pair that generates a 189 bp fragment.[28] Thirty eight cycles of PCR were performed for 45 seconds at 95°C, 35 seconds at 55°C, and 75 seconds at 72°C. An aliquot of the PCR product was digested overnight with *Hin*fI restriction endonuclease (for detecting *MTHFR*)[9] or with *Hae*III (for detecting *MS*).[28] The reaction products were subjected to electrophoresis using a 1.8% agarose gel. The homozygous normal *MTHFR* and *MS* alleles are not digested by their respective restriction endonucleases, whereas the polymorphisms create recognition sequences which are digested. The non-mutated (Ala/Ala) *MTHFR* PCR product is 198 bp long. The homozygous mutant (Val/Val) allele is thus digested completely by the enzyme and gives a 175 bp fragment and a 23 bp fragment. The latter runs off the gel and is not visible after electrophoresis. The heterozygous genotype (Ala/Val) yields both the 198 and 175 bp fragments upon *Hin*fI digestion. Similarly, the non-mutated (Asp/Asp), homozygous mutant (Gly/Gly), and heterozygous (Asp/Gly) genotypes of *MS* yield 189, 159, and 189 plus 159 bp bands, respectively.

As shown in table 1, we found that the C677T Ala/Val or Val/Val *MTHFR* genotype was more prevalent in NTD cases than in controls. When Ala/Val and Val/Val genotypes were combined, 48% of NTD cases had both alleles, compared with 17% of controls. There was a 4.0-fold increased risk for NTDs in fetuses having the Ala/Val genotype (95% confidence interval 1.8-8.8), and the risk increased to 6.6-fold in fetuses with the Val/Val genotype (95% confidence interval 1.3-24.9). The fetal *MS* A2756G Gly/Gly and Asp/Gly genotypes were not associated with risk of NTD (table 1). Furthermore, we also found no association between combined *MTHFR* and *MS* polymorphisms and risk of NTDs (table 2).

Table 2 Odds ratios and 95% confidence intervals of 5,10-methylenetetrahydrofolate reductase (MTHFR) and methione synthase (MS) genotypes in fetuses with NTDs (cases) and controls

MTHFR genotype	MS genotype	NTDs (cases)	Controls	Odds ratio	95% confidence interval
Ala/Ala	Asp/Asp	32	53	1.0	
	Asp/Gly	8	9	1.5	0.5–4.2
	Gly/Gly	0	1	—	—
Ala/Val	Asp/Asp	22	10	1.0	
	Asp/Gly	7	1	3.2	0.3–29.4
	Gly/Gly	0	0	—	—
Val/Val	Asp/Asp	5	1	1.0	
	Asp/Gly	3	1		
	Gly/Gly	0	0	—	—

Our findings using amniotic fluid cells indicate that fetuses homozygous or heterozygous for the C to T substitution in the *MTHFR* gene are at increased risk for NTDs. In order to compare our results to those of others, we summarised the *MTHFR* genotype frequencies in previously reported NTD cases from all studies with ⩾50 subjects (table 3). We selected studies with NTD cases exceeding 50 subjects. Our study, reporting the *MTHFR* genotype of 82 NTD cases and 76 controls, is the fourth largest study in terms of the number of NTD cases. Our results are similar to those of previous studies which suggested that either homozygosity or heterozygosity for the C677T mutation in the fetal *MTHFR* gene is a risk factor for NTDs. A meta-analysis of the available data indicated that homozygosity for the C677T mutation resulted in an approximately two-fold increase of risk for NTDs.[11] In the study reported here, we found that NTD risk was increased in fetuses having the Val/Val and Ala/Val genotypes. When compared to previous studies, our odds ratios for both heterozygous and homozygous *MTHFR* C677T genotype frequencies in NTD cases are higher than those previously reported. The mechanism accounting for the higher ratios is unknown, but may relate to the ethnic or geographical composition or the folate nutriture of our study population. Folate nutriture is mentioned here because previous studies have shown that folic acid supplementation decreases the incidence of NTDs.[2 3]

Our finding of no association between the A2756G (Gly/Gly) polymorphism in the *MS* gene and the occurrence of the NTD phenotype is similar to that of van der Put *et al*,[30] who reported that there is no increased prevalence of the Asp/Gly or Gly/Gly genotypes in fetuses with NTDs or their mothers. They found that the prevalence in controls of the Asp/Asp, Asp/Gly, and Gly/Gly genotypes was 71%, 26%, and 3%, respectively, which is similar to our values of 84%, 14%, and 3% in controls presented here. Shaw *et al*[31] and Morrison *et al*[32] also reported that overall percentages of Asp/Gly and Gly/Gly were not increased in infants with NTDs. Christensen *et al*[33] presented data suggesting that the homozygous mutant genotype for the A2756G polymorphism in methionine synthase was associated with a reduced risk for NTD in children. Given the importance of MS in homocysteine metabolism, it is tempting to speculate that during the course of evolution, some mutations of *MS* were so deleterious that they were lethal to the fetus and were thus not propagated.

To our knowledge, this is the first reported study of interactions between frequently occurring polymorphisms of two genes involved in folate metabolism. We did not find strong associations between *MTHFR* and *MS* polymorphisms and the risk of NTDs. van der Put *et al*[34] recently hypothesised that combined heterozygosity for two common *MTHFR* mutations may be an additional risk factor for NTDs. The significance, if any, of the weak *MS*/*MTHFR* associations that we observed in this study requires further evaluation, and possible confounding factors resulting from genetic associations involved in folate metabolism as well as folate nutriture may warrant further investigation.

Table 3 Heterozygous and homozygous MTHFR C677T genotype frequencies in published NTD cases

No of NTD cases	No heterozygous (%)	No homozygous (%)	Homozygous odds ratio	95% confidence interval	Reference
214	100 (47)	41 (19)	Not reported	—	12
153	Not reported	29 (19)	2.6	1.4–4.8	16
137	60 (44)	19 (14)	Not reported	—	19
82	32 (39)	15 (18)	3.5	1.3–9.4	14
56	26 (46)	11 (20)	2.2	0.8–6.0	26
55	26 (47)	7 (13)	2.9	1.0–7.9	15
82	30 (37)	9 (11)	6.6	1.3–24.9	Our study

In conclusion, our data support previous studies suggesting that the C677T mutation in *MTHFR* is associated with increased risk for NTDs. Although a common polymorphism in the *MS* gene was not a strong risk factor for NTDs, associations between *MTHFR* and *MS* polymorphisms slightly increased the risk. Further research is warranted to evaluate comorbidity of *MTHFR* and *MS* polymorphisms in a large population.

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Expression of HCM causing mutations: lessons learnt from genotype-phenotype studies of the South African founder *MYH7* A797T mutation

EDITOR—Genotype-phenotype correlations provide another perspective in studies seeking to identify the factors that underlie the clinical variability that is a feature of several inherited diseases. This approach has been particularly revealing in investigations into the molecular causes and phenotypic heterogeneity associated with hypertrophic cardiomyopathy (HCM), a common inherited primary cardiac disorder.[1 2] Although, as its name suggests, hypertrophy may be a noticeable feature of the disease, it is not invariant, nor does the degree of hypertrophy necessarily correlate with the risk of sudden cardiac death (SCD), which is the most feared consequence of HCM.[3 4]

Molecular genetic investigations have shown that HCM is caused by more than 100 distinct mutations in at least seven different sarcomeric protein encoding genes.[5] When the clinical features of HCM are correlated in a family context with the specific disease causing gene and its associated mutation, a recognisable pattern emerges. Essen-