Gene–gene interaction in folate-related genes and risk of neural tube defects in a UK population

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Objective: To investigate the contribution of polymorphic variation in genes involved in the folate-dependent homocysteine pathway in the aetiology of neural tube defects (NTD).

Design: Case-control association study.

Subjects: A total of 530 individuals from families affected by NTD, 645 maternal controls, and 602 healthy newborns from the northern UK.

Main outcome measures: Seven polymorphisms in six genes coding for proteins in the folate-dependent homocysteine pathway (MTHFR 677C→T, MTHFR 1298A→C, MTRR 66A→G, SHMT 1420C→T, C/S 844ins68, GCPII 1561C→T, RFC-1 80G→A). The interaction of each polymorphism and the effect of gene–gene interactions (epistasis) upon risk of NTD were assessed using logistic regression analysis.

Results: The MTHFR 677C→T polymorphism was shown to represent a risk factor in NTD cases (CC vs CT+TT odds ratio [OR] 2.03 [95% confidence interval (CI) 1.09, 3.79] p = 0.025) and the MTRR 66A→G polymorphism was shown to exert a protective effect in NTD cases (AA vs AG+GG OR 0.31 [95% CI 0.10, 0.94] p = 0.04). When statistical tests for interaction were conducted, three genotype combinations in cases (MTRR/GCPII, MTHFR 677/C/S, MTHFR 677/MTRR) and one combination in case mothers (C/S/RFC-1) were shown to elevate NTD risk. Maternal–fetal interaction was also detected when offspring carried the MTHFR 677C→T variant and mothers carried the MTRR 66A→G variant, resulting in a significantly elevated risk of NTD.

Conclusion: Both independent genetic effects and gene–gene interaction were observed in relation to NTD risk. Multi-locus rather than single locus analysis might be preferable to gain an accurate assessment of genetic susceptibility to NTD.

F ailure of closure of the developing neural tube leads to a number of related conditions collectively termed neural tube defects (NTD). The precise aetiology of such conditions is not known, but can involve a number of environmental and genetic factors. NTD is known to have a significant genetic component, with multiple cases known to occur within a single family. A common genetic basis is assumed but since various forms or degrees of severity of NTD have been observed in a single family, susceptibility is probably dictated by a polygenic mode of inheritance as well as being modulated by environmental exposures.

Significantly, mothers of NTD affected offspring typically display lower than normal plasma folate levels and an elevation in homocysteine (Hcy) levels, indicating that defects in the folate-dependent Hcy pathway may play an important role in the aetiology of these conditions. It is also now well accepted that periconceptional supplementation with folic acid substantially decreases the risk of occurrence and recurrence (by up to 70%) of neural tube defects (reviewed by Lucock). These observations have resulted in considerable interest in the study of genetic polymorphisms in loci of the folate-dependent Hcy metabolic pathway as risk factors for NTD, as a functional defect at any such locus could impact upon the distribution of folate, Hcy, and their intermediates.

Many enzyme-coding loci of the folate-dependent Hcy pathway have now been screened for mutations. The first such polymorphism identified as a risk factor for NTD was the C→T mutation at position 677 of the MTHFR gene. Additional polymorphisms in alternative loci involved in folate metabolism, and folate transport, have since been examined as potential risk factors. Although individual polymorphisms have been implicated as NTD risk factors for certain populations, as yet no single polymorphism in any candidate gene has been implicated as a risk factor in all studies. Digenic inheritance, in which mutations in each of two unlinked genes results in the disease phenotype, has been implicated in numerous diseases, including the NTD related holoprosencephaly. The interconnected nature of the folate-dependent Hcy pathway is possible that particular combinations of genetic variants may underlie NTD susceptibility. However, as yet no association between combinations of genetic variants and NTD has been reported. The creation of a human genome single nucleotide polymorphism (SNP) map has highlighted the frequency with which SNPs occur across the genome. With an SNP being present every 1000 bases (on average) it is probable that no two individuals will possess the same allelic combination. By virtue of this, it is likely that critical combinations of variant alleles will be responsible for increasing disease susceptibility. In the case of NTD it is hypothesised that a mutation load in folate-related Hcy metabolism genes which, in some instances, cannot be overcome by exogenous nutrient supplementation, might be sufficient to cause NTD. Since few studies examine multiple polymorphisms, it may also be that the crucial gene–gene

Abbreviations: Hcy, homocysteine; NTD, neural tube defects; SNP, single nucleotide polymorphism; TDT, transmission disequilibrium testing
interaction is, as yet, unexamined. As such, it is important to not only measure allele frequencies of candidate gene polymorphisms in new NTD cohorts, but to examine the impact of new combinations of loci in each population studied. Here we examine a suite of seven polymorphisms in six folate pathway genes for their impact on NTD risk in a collection of NTD patients and families, with controls, from the northern UK, where NTD prevalence is relatively high at a rate of 17.9 per 10 000 births.12

METHODS

Subjects

The study population consisted of individuals with NTDs (n = 219), mothers of NTD cases (n = 211), and where both case and mother were present, fathers were invited to participate (n = 100) in order to compile case–parent triads. Of the total study group, 126 case mother–child pairs and 275 control mother–child pairs were available for analysis, and in all other instances only the mother or child were enrolled onto the study. Recruitment took place throughout the northern region of the UK between 1993 and 2002 through the Northern Genetics Service. Participants were visited by a genetic research nurse who gained informed consent prior to obtaining a family history, completing a questionnaire with the mother of the affected individual, and taking a venous blood sample, or buccal swab sample, from family members for DNA extraction. Public awareness of the research study was raised with local media assistance and liaison with the Association for Spina Bifida and Hydrocephalus (ASBAH). Information about the type of defect was obtained from 203 of the NTD cases: 109 (53.7%) spina bifida, 51 (25.1%) spina bifida occulta, 32 (15.8%) anencephaly, and 11 (5.4%) other NTD. In addition, information was available in 200 of 223 cases pertaining to the affected status of offspring born to case mothers (multiple affected pregnancies were included): 126 (63.0%) spina bifida, 39 (19.5%) spina bifida occulta, 31 (15.3%) anencephaly, and four (2.0%) other NTD. Only those cases with an unequivocal spina bifida occulta diagnosis, displaying clear signs such as haemangioma, significant dimple, hairy patch, or associated neurological impairment were included in the study cohort. X-ray evidence was obtained in some instances. The control population was drawn from the North Cumbria Community Genetics Project (NCCGP).13 The NCCGP is a DNA and tissue bank consisting of samples collected consecutively between 1996 and 2003. Cord blood was collected from newborns from 1996 and from 1999, mothers were asked for their consent to retain antenatal blood samples. Over 7000 infant samples and 3000 maternal samples have been collected representing over 85% of births at West Cumberland Hospital, Whitehaven, Cumbria, UK. The control group were Caucasian women of childbearing age and did not differ ethnically/racially from Cumbria, UK. The control group were Caucasian women of antenatal blood samples. Over 7000 infant samples and Cord blood was collected from newborns from 1996 and from 2003. MREC and West Cumbria LREC. This study was obtained from the Northern and Yorkshire MREC and West Cumbria LREC.

Genetic analyses

DNA was extracted from either blood samples or mouth-swabs using standard techniques. Seven polymorphisms in six folate pathway genes were examined by PCR-RFLP. Table 1 lists the polymorphisms examined and reaction conditions employed for amplification.14–21 Not all loci were amplified from every subject due to problems with DNA quality, quantity, and PCR amplification.

Statistical analyses

Hardy-Weinberg tests on genotypic data were conducted using the method of Guo and Thompson.22 The method of Schaid and Jacobsen23 was applied where appropriate to correct for deviation from Hardy-Weinberg equilibrium (HWE) (that is if HWE was not observed). Logistic regression modeling of all seven loci was undertaken to investigate both individual locus effects and gene–gene interaction in cases and case mothers. In addition, multivariate logistic regression analysis of joint mother–child genotypes was applied to determine whether maternal–fetal interaction was significantly influencing disease risk. For each locus the wild-type genotype was compared to pooled heterozygote and homozygote variants. Results were represented as odds ratios with 95% confidence intervals (CI). Statistical analysis was undertaken using the STATA software package. Transmission disequilibrium testing (TDT) was undertaken using the method of van den Oord et al24 and incorporated data from both complete and incomplete trios (n = 124).

RESULTS

The number of individuals and genotype frequency for each of the seven studied polymorphisms are presented in Table 2. Genotypes at MTRR were not in Hardy-Weinberg equilibrium (HWE) for NTD cases, case fathers, or control infants (table 2) but all other comparisons were within HWE.

A case-control comparison of individual loci was conducted for NTD cases using a multivariate logistic regression model (table 3). After adjusting for all other genotypes, significant effects were observed which were independent of other genotypes. The MTHFR 677C–T polymorphism was shown to represent a significant risk factor in NTD cases (CC vs CT+TT OR 2.03 [95% CI 1.09, 3.79]) and MTRR 66A–G conferred a protective effect in the same group (AA vs AG+GG OR 0.31 [95% CI 0.10, 0.94]). When mothers of NTD offspring were considered as the “case” phenotype a case-control analysis of individual locus genotypes produced no significant results (table 3).

Logistic regression analysis of all seven loci for evidence of interaction showed three genotype combinations to confer a risk of NTD in cases: GCPII 1561C–T × MTRR 66A–G (p = 0.004); MTHFR 677C–T × MTRR 66A–G (p = 0.003); and MTHFR 677C–T × CBS 844ins68 (p = 0.007). One combination of genotypes was shown to represent a significant risk factor for NTD pregnancy: RFC-1 80G–A × CBS 844ins68 (p = 0.014).

Analysis of all combinations of mother–child genotypes for maternal–fetal interaction produced only one statistically significant result: maternal MTRR 66A–G × fetal MTHFR 677C–T (p = 0.001).

Transmission disequilibrium testing (TDT) was undertaken using both complete and incomplete trios (n = 124) to ascertain whether any alleles were preferentially transmitted from parents to affected offspring. No significant observations were detected.

DISCUSSION

NTD is recognised to have a complex aetiology, involving both environmental and genetic factors. Although enzymes involved in the folate-dependent Hcy pathway are strongly implicated in NTD, the numerous candidates and wide ethnic and population level differences in allele frequencies of their polymorphic variants10 11 suggest that no single genetic factor is likely to be responsible for NTD. Estimation of allele frequencies in new sample sets and examination of potential gene–gene interactions must therefore be undertaken in several independent populations. Here we have undertaken a large-scale analysis of seven single nucleotide polymorphisms in six candidate genes involved in the folate-dependent Hcy

<table>
<thead>
<tr>
<th>Locus*</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>PCR conditions</th>
<th>Buffer†</th>
<th>Restriction endonuclease</th>
<th>Gel/ABI‡</th>
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</thead>
<tbody>
<tr>
<td>MTHFR 677C→T</td>
<td>5′-TGA AGG AGA AGG TGT</td>
<td>5′-TGA GAG TGG GGT</td>
<td>33°C (96°C 30 s)</td>
<td>V</td>
<td>Hinf I</td>
<td>ABI</td>
</tr>
<tr>
<td>CTG CGG AA-3</td>
<td>GGA GGG AGC TT-3</td>
<td>61°C (30 s, 72°C 30 s)</td>
<td>V</td>
<td>Mbo II</td>
<td>ABI</td>
<td></td>
</tr>
<tr>
<td>MTHFR 1298A→C</td>
<td>5′-CTT TGG GCT GAA</td>
<td>5′-CAG GGG ATG AAC</td>
<td>38°C (95°C 30 s)</td>
<td>V</td>
<td>Nde I</td>
<td>ABI</td>
</tr>
<tr>
<td>GGA CTA CTA C-3</td>
<td>CAG GTC GCC C-3</td>
<td>59°C (30 s, 72°C 30 s)</td>
<td>V</td>
<td>–</td>
<td>Gel</td>
<td></td>
</tr>
<tr>
<td>MTRR 66A→G</td>
<td>5′-GCA AAG GGC ATC GCA</td>
<td>5′-CAC TTC CCA ACC</td>
<td>30°C (94°C 30 s)</td>
<td>V</td>
<td>–</td>
<td>Gel</td>
</tr>
<tr>
<td>GAA GAC AT-3</td>
<td>AAA ATT CIT CAA AG-3</td>
<td>55°C (30 s, 72°C 30 s)</td>
<td>V</td>
<td>–</td>
<td>Gel</td>
<td></td>
</tr>
<tr>
<td>GCPII C1561T</td>
<td>5′-CAT TCT GGG ATT AAT</td>
<td>5′-AAA CAC CAC CTA</td>
<td>35°C (95°C 30 s)</td>
<td>I</td>
<td>Acc II</td>
<td>Gel</td>
</tr>
<tr>
<td>TTA GCA-3</td>
<td>TGT TTA AAG CTA-3</td>
<td>48°C (30 s, 72°C 30 s)</td>
<td>I</td>
<td>–</td>
<td>Gel</td>
<td></td>
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<tr>
<td>SHMT 1420C→T</td>
<td>5′-AGA GGT CAA GGA GAG</td>
<td>5′-TTT GCC CTA CAC</td>
<td>35°C (94°C 30 s)</td>
<td>V</td>
<td>Ear I</td>
<td>Gel</td>
</tr>
<tr>
<td>AGC GCC AG-3</td>
<td>CAT CT C-3</td>
<td>56°C (30 s, 72°C 30 s)</td>
<td>V</td>
<td>–</td>
<td>Gel</td>
<td></td>
</tr>
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</table>

**Table 2** Genotype frequencies, allele frequencies, and tests for comparson to Hardy-Weinberg equilibrium at seven candidate loci

A clear association between MTHFR 677C→T genotype and NTD risk was observed in the NTD case population investigated in this study (CC vs CT+TT OR 2.03 [95% CI 1.09, 3.79]). A number of previous studies have established the common 677C→T variant of MTHFR as an independent risk factor associated with NTD.46 Although several
subsequent studies in other populations have been unable to confirm this finding.\textsuperscript{25–30} a meta-analysis conducted by Botto and Yang\textsuperscript{30} identified an overall odds ratio of 1.75 [95% CI 1.14, 2.18] in NTD cases. Previous studies have also shown MTHFR 677C–T to represent a risk factor in mothers of NTD cases.\textsuperscript{10} However, in our NTD case mother population, the 677C–T variant was not shown to confer any risk. Indeed, no individual locus in mothers of NTD cases was shown to significantly influence risk of NTD pregnancy in our cohort.

Since both hyperhomocysteinemia and elevated vitamin B\(_\text{12}\) levels are independent indicators of NTD risk,\textsuperscript{31} some attention has focused on methionine synthase, an enzyme which catalyses the vitamin B\(_\text{12}\) dependent remethylation of Hcy to methionine, as a likely NTD risk factor. However, the MS 2756A–G polymorphism has not been linked to NTD in British cases,\textsuperscript{25,32} although Doolin et al\textsuperscript{32} have recently suggested that this polymorphism may be a risk factor via the maternal line. Since methionine synthase reductase (MTRR) is involved in maintaining MS in an active state, it is also seen as a prime candidate. The results of this study suggest that the MTRR 66A–G polymorphism acts to protect against NTD but only in cases (p = 0.04) and not case mothers (p = 0.08). This could be interpreted as the A allele representing the risk allele in our study population. In a previous study Wilson et al hypothesised that the 66G allele represented a protective effect (OR 0.31 [95% CI 0.10, 0.94]) as it is in this study. Wilson et al\textsuperscript{33} detected a significant increase in risk of NTD for carriers of the MTRR 66G allele when cobalamin levels were low, or when combined with the MTHFR 677C–T mutant genotype, and more recently Zhu et al reported an association between the G allele and NTD risk in a US population.\textsuperscript{34} However, Lucock et al\textsuperscript{35} were unable to implicate MTRR 66A–G as a NTD risk factor in a UK population. We see a strongly reduced risk of NTD associated with the 66G allele of MTRR in our case population. These results must be interpreted with caution due to the observed deviation from HWE of MTRR genotypes in the NTD case group. The current study detected an interaction between MTHFR 677C–T and MTRR 66A–G in NTD cases (p = 0.003), which supports the observations of Wilson et al.\textsuperscript{33} A maternal (MTRR 66A–G)–fetal (MTHFR 677C–T) interaction which elevated NTD risk was also observed (p = 0.001).

This study investigated the influence of gene–gene interaction on the risk of NTD in a sizeable population using multivariate logistic regression analysis to implicate a number of genes as risk factors for NTD. Previous studies have indicated that the possession of more than one polymorphism at folate-related loci can elevate risk of NTD.\textsuperscript{35–36} The chosen method of analysis in this study did not involve a pair-wise analysis of genotype combinations but a logistic regression model to incorporate all seven loci.

Epistasis, or the interaction between genes, is a phenomenon of increasing importance in the field of genetic epidemiology. As a statistical concept, analysis of epistasis can lead to improved power for the detection of genetic effects.\textsuperscript{37–38} There is however some discussion as to the differing use of the term epistasis in a statistical or biological context. It has been adopted here as a statistical term with the caveat that it can be difficult to infer biological meaning from quantitative data measuring disease risk as the outcome.\textsuperscript{39–40} With regard to polymorphisms in the folate-dependent Hcy metabolic pathway, a substantial amount is known about the biological role of the candidate genes under investigation, therefore a plausible biological model could be postulated for the epistatic events observed. The benefit of the analysis of epistasis therefore does not lie in further elucidating biological mechanisms underlying disease pathogenesis, but in determining the true contribution of genetic factors to disease susceptibility.

As discussed above, when tested independently, the MTHFR 677C–T polymorphism represents a risk factor (OR 2.03 [95% CI 1.09, 3.79] p = 0.025) and the MTRR 66A–G polymorphism, a protective effect (OR 0.31 [95% CI 0.10, 0.94]). Furthermore, when found in combination, these variants significantly influence NTD risk in NTD cases. In addition, the MTHFR 677C–T variant in combination with the C677T allele of MTHFR is a significant risk factor (OR 0.65 [95% CI 0.36, 1.14]). The benefit of the analysis of epistasis therefore does not lie in further elucidating biological mechanisms underlying disease pathogenesis, but in determining the true contribution of genetic factors to disease susceptibility.

It is clear that maternal genotype can impact upon pregnancy outcome,\textsuperscript{41–43} with further influence possible from maternal–fetal interaction.\textsuperscript{44–46} Christensen et al reported a notable elevation in NTD risk, from a non-significant risk in individual cases or mothers, when the MTHFR 677C–T variant was harboured by both mother and offspring (odds ratio (OR) for TT/TT genotype combination: 6.00 [95% CI 1.26, 28.53]).\textsuperscript{47} Although this finding is not replicated using the statistical model applied in the context of this study, one
example of statistically significant maternal–fetal interaction was demonstrated.

In summary, MTHFR 677C→T has been shown to represent a risk factor in NTD cases in a UK population. Furthermore, when interactions between this and other polymorphisms have been investigated, significant interactions have been observed. Molecular genetic advances are resulting in single locus investigations being superseded by multi-locus analyses, however interpretation of data is consequently complex. A significant challenge remains in translating the observations of association studies, such as those presented in this paper, to a clinical setting where NTD families are counselled with regard to genotype derived susceptibility.

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