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Evaluation of Folate Metabolism Gene Polymorphisms as Risk Factors for Open and Closed Neural Tube Defects

K. Doudney,[1,2] J. Grinham,[1] J. Whittaker,[3] S.A. Lynch,[4] D. Thompson,[5] G.E. Moore,[1] A.J. Copp,[1] N.D.E. Greene,[1] and P. Stanier[1]*

[1]UCL Institute of Child Health, University College London, London, UK

[2]Department of Pathology, University of Otago, Christchurch, New Zealand

[3]Department of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London, UK

[4]National Centre for Medical Genetics, Our Lady's Children's Hospital Crumlin, Dublin, Ireland

[5]Department of Neurosurgery, Great Ormond Street Hospital for Children, London, UK

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TO THE EDITOR:

Neural tube defects (NTDs) are among the commonest human birth defects, affecting around 1 per 1,000 births [Copp et al., 2003]. A genetic basis for NTDs is implicated based on the high recurrence risk within families compared to the general population occurrence rate [Carter, 1974]. Although many mouse models for NTD demonstrate Mendelian inheritance, the etiology of human NTDs appears more complex, involving a combination of genetic risk factors and environmental triggers [Harris and Juriloff, 2007]. Periconceptional folic acid supplementation has been shown to be extremely effective at reducing the incidence of NTDs [MRC Vitamin Study Research Group, 1991]. Sub-optimal maternal dietary folate status may be associated with increased risk of a NTD in an offspring [Kirke et al., 1993]. Polymorphic variants in some of the key steps of folate metabolism have been shown to diminish its bioavailability or bioactivity. One of the most frequently cited is the Dutch study showing increased risk for infants affected with spina bifida who were homozygous for the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) 677C > T thermolabile polymorphism (A222V) [van der Put et al., 1995]. It has also been estimated that 12% of all fetuses with NTDs in Ireland are attributable to the same variant [Kirke et al., 1996], although several other studies have failed to detect this association [Rampersaud et al., 2003; Amorim et al., 2007].

Other variants implicated in increased NTD risk, include a second *MTHFR* variant 1298A > C [van der Put et al., 1998], which also reduces enzyme activity though to a lesser extent than 677C > T. Similarly, *MTHFD1* 1958G > A (R653Q) [Brody et al., 2002], *MTR* 2756A > G (D919G) [Doolin et al., 2002], *MTRR* 66A > G (I22M) [Doolin et al., 2002], a 19 bp deletion in dihydrofolate reductase (*DHFR*) intron 1 [Johnson et al., 2004] and a frequent variant (80G > A; R27H) in the reduced folate carrier (*RFC1*) [Chango et al., 2000; De Marco et al., 2002], have been implicated in elevated risk, particularly when occurring in combination with *MTHFR* variants [Guéant-Rodriguez et al., 2003; Shang et al., 2007]. Nevertheless, numerous studies fail to replicate these associations, suggesting that geographical, ethnic differences or in some cases, study design leading to either type I or type II errors, may also play a role in this variability [Botto and Yang, 2000; Rampersaud et al., 2003; Boyles et al., 2005; Amorim et al., 2007].

While the majority of studies report on patients with defects in primary neurulation (anencephaly or spina bifida aperta), there has been less focus on low sacral and coccygeal NTDs resulting from failed or incomplete secondary neurulation. These anomalies are often skin covered and described as "spina bifida occulta" when asymptomatic or "occult spinal dysraphism" (OSD) when symptomatic. In OSD, lesions include lipomas of the terminal spinal cord and filum terminale, dermal sinus tracks and diastematomyelia or split cord malformations. Lower limb weakness, pain, foot

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*Correspondence to:
P. Stanier, UCL Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK. E-mail: pstanier@ich.ucl.ac.uk
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deformity, and impaired sphincter function through disturbed neurological function are common. Spinal cord tethering often contributes to these symptoms and typical treatment is to untether the low spinal cord. The prevalence of OSD is thought to be around two per thousand live births [Campbell, 1977]. Unlike open NTDs, this group of defects is often not immediately recognized, being diagnosed either at the onset of symptoms or incidentally through treatment of unrelated problems [Bui et al., 2007]. It is important to note that, while 85% of fetuses with open spina bifida in the UK are terminated, closed forms of spina bifida are not diagnosed prenatally, with the majority of infants surviving birth. Occult spinal dysraphism is therefore an important cause of morbidity and neurosurgery during childhood.

In this study, we investigated the role of *MTHFR*, *MTHFD1*, *DHFR*, *MTR*, *MTRR*, and *RFC1* polymorphisms in OSD cases and controls. Patients (n = 103) with symptomatic OSD were recruited at the Great Ormond Street Hospital neurosurgical clinic. Normal controls (n = 192) were randomly selected from a large collection of volunteers with full details of pregnancy history as described previously [Apostolidou et al., 2007], which were recruited from the North Thames Regional Health Authority. Comparison was then made to patients with open NTDs (126 spina bifida aperta and 49 with anencephaly recruited from either the Newcastle-upon-Tyne or North Thames Regional Health Authorities between 1993 and 2006). Patients and controls were White European and identified as consecutive consenting volunteers at the respective clinics. All samples were collected with informed consent and study approval was granted from the respective Research Ethics Committees.

Genotyping was performed by DNA sequencing of PCR products amplified using primers flanking *MTHFR* (rs1801133), *MTHFR* (rs1801131), *MTHFD* (rs2236225), *MTR* (rs1805087), *MTRR* (rs1801394), *DHFR* (19 bp indel), or for *RFC1* (rs1051266) by RFLP analysis essentially as described by Chango et al. [2000]. Primer sequences are available on request. The observed distribution of genotypes among patients and controls were consistent with Hardy–Weinberg equilibrium (data not shown). The distribution of allele frequencies for each of the polymorphisms is given in Table I. Odds ratios and 95% confidence intervals were calculated to estimate the relative risk for different genotype combinations in cases versus controls. Analysis was based on two models, either dominant, in which the effects of both homozygotes and heterozygotes are equal, or recessive, in which effects are expected only from homozygotes. Fisher's exact test was used to calculate *P* values. We did not detect an association between any of the genotypes and increased risk of OSD. In contrast, for polymorphisms in *MTHFR* and *MTRR* we detected significant differences in allele frequencies between controls and patients with open NTDs. The *MTHFR* 677TT genotype was associated with a reduced risk for all open (OR 0.57; $P = 0.038$) but not closed NTDs. When NTD patients were sub-divided, this association was still apparent for spina bifida aperta (OR 0.46; $P = 0.033$), but was not significant for anencephaly. Surprisingly, these data indicate a protective effect of the 677T allele rather than an increased risk, that is, a lower frequency among NTD patients. Recessive inheritance of *MTRR* 66GG was associated with increased risk when all NTDs were considered together (total NTDs), with an OR of 1.47 ($P = 0.048$). However, this effect did not reach statistical significance when NTDs were sub-divided. Similarly, there was a trend towards increased risk of open NTDs for the *RFC1* 80AA genotype but this did not reach statistical significance (OR 1.46; CI 0.92–2.32; $P = 0.07$). When corrected for multiple testing, none of these *P* values reached significance. Larger control and patient sample numbers will be required to confirm and extend these findings. Studies of this type are frequently limited in power by the number of available cases and controls and it could be argued that only modest to large effects can be detected. Nevertheless, the cohorts reported here would be expected to detect considerably weaker associations than those described for MTHFR in the Dutch and Irish populations [van der Put et al., 1995; Kirke et al., 1996].

To investigate a possible effect of folate pathway gene–gene interactions, we stratified the cases and controls by genotype. Since this analysis generates many potential combinations, none of the possible associations survive correction for multiple testing. However, the following associations detected in the absence of correction might suggest interactions that should be more specifically investigated in the future. Notably, increased risk of open NTDs was detected for combinations of *MTHFR* 677CT with *DHFR* inin (OR 2.57, $P = 0.005$) and *RFC1* 80GG (OR 2.45, $P = 0.015$); and *MTRR* 66GG with *RFC1* 80GG (OR 3.3, $P = 0.019$). Apparent reductions in risk of NTDs were noted for the double heterozygote combination of *MTHFR* 677CT with *DHFR* indel (OR 0.5, $P = 0.02$). Analysis of genotype combinations in OSD revealed an apparent increased risk associated with the combined presence of *MTHFR* 677TT with *DHFR* deldel (OR 5.43, $P = 0.002$) or decreased risk for *MTHFR* 677CT with *RFC1* 80AG (OR 0.33, $P = 0.003$) and *MTRR* 66AG with *RFC1* 80AG (OR 0.33, $P = 0.005$).

A modest increase in risk for NTDs has previously been reported for *MTHFR* 677TT infants of mothers not using vitamin supplements, compared to those who do [Shaw et al., 1998]. A similar finding was found for conotruncal heart defects in *RFC1* 80GG infants, but not in a similar group with orofacial defects [Shaw et al., 2003]. While it is possible that both dietary intake and circulating folate status may have had an important effect on the influence of the various folate pathway enzyme variants in this study, it was not possible to take this into account due to incomplete data. Nevertheless, from those patients where data are available, it should be noted that the great majority did not use any supplements.

In a previous study, we investigated the hypothesis that an inborn error of folate metabolism in the fetus itself may be causally linked to NTDs using a simple test of folate metabolism in patient cell lines [Dunlevy et al., 2007]. This data showed that there may be an underlying defect present in a subset of NTDs, although this did not appear to be directly caused by the commonly analyzed "risk factors" such as *MTHFR* C677T [Dunlevy et al., 2007]. The current study focuses for the first time on a distinct group of NTD samples with OSD, who, along with a cohort of open NTD patients, do not indicate a role for the widely reported polymorphisms in folate cycle enzymes in their etiology. Instead, it is reasonable to suggest that the causative genetic factors in these patients have not yet been identified or that multigenic interactions such as those investigated here are responsible for the genetic component of NTD prevalence. This latter possibility will either require a much larger sample size to

TABLE I. Risk of Open or Closed NTDs Associated With Folate Pathway Gene Variants

	Genotypes, n			Recessive	Dominant
MTHFR 677C-T	**CC**	**CT**	**TT**	**OR (95% CI)**	**OR (95% CI)**
Control	73	86	28	Reference group	Reference group
OSD	39	39	11	0.80 (0.38–1.69)	0.82 (0.49–1.37)
SB aperta	49	63	9	0.46 (0.21–1.00)[a]	0.94 (0.59–1.50)
Anencephaly	22	20	5	0.68 (0.25–1.86)	0.73 (0.38–1.39)
Open NTDs	71	83	14	0.52 (0.26–1.02)[b]	0.87 (0.57–1.34)
Total NTDs	124	137	26	0.57 (0.32–0.99)[c]	0.72 (0.50–1.04)[d]

	Genotypes, n			Recessive	Dominant
MTHFR 1298 A-C	**AA**	**AC**	**CC**	**OR (95% CI)**	**OR (95% CI)**
Control	89	68	19	Reference group	Reference group
OSD	42	40	10	1.01 (0.45–2.27)	1.22 (0.73–2.02)
SB aperta	55	45	10	0.83 (0.37–1.85)	1.02 (0.64–1.65)
Anencephaly	17	22	5	1.06 (0.37–3.01)	1.62 (0.83–3.19)
Open NTDs	72	77	15	0.89 (0.48–1.71)	1.31 (0.85–2.00)
Total NTDs	127	121	25	0.83 (0.44–1.56)	1.18 (0.80–1.72)

	Genotypes, n			Recessive	Dominant
MTHFD1 1958 G-A	**GG**	**GA**	**AA**	**OR (95% CI)**	**OR (95% CI)**
Control	71	82	33	Reference group	Reference group
OSD	38	34	20	1.29 (0.69–2.40)	0.88 (0.53–1.46)
SB aperta	37	59	23	1.11 (0.62–2.00)	1.37 (0.84–2.23)
Anencephaly	14	22	9	1.16 (0.51–2.64)	1.37 (0.68–2.74)
Open NTDs	51	81	32	1.12 (0.66–1.93)	1.37 (0.88–2.13)
Total NTDs	95	133	58	1.18 (0.73–1.89)	1.24 (0.84–1.82)

	Genotypes, n			Recessive	Dominant
DHFR del +/−	**++**	**+−**	**−**	**OR (95% CI)**	**OR (95% CI)**
Control	52	103	35	Reference group	Reference group
OSD	26	47	19	1.15 (0.62–2.15)	0.96 (0.55–1.67)
SB aperta	33	64	16	0.73 (0.38–1.39)	0.91 (0.55–1.53)
Anencephaly	16	21	10	1.2 (0.54–2.63)	0.73 (0.37–1.44)
Open NTDs	49	85	26	0.86 (0.49–1.50)	0.85 (0.54–1.36)
Total NTDs	84	146	52	1.00 (0.62–1.61)	0.89 (0.59–1.34)

	Genotypes, n			Recessive	Dominant
MTR 2756A-G	**AA**	**AG**	**GG**	**OR (95% CI)**	**OR (95% CI)**
Control	118	60	10	Reference group	Reference group
OSD	52	35	1	—	1.17 (0.70–2.00)
SB aperta	80	36	4	0.61 (0.19–2.00)	0.84 (0.52–1.36)
Anencephaly	22	13	3	1.53 (0.40–5.83)	1.23 (0.60–2.49)
Open NTDs	102	49	7	0.83 (0.31–2.22)	0.93 (0.60–1.44)
Total NTDs	147	76	8	0.64 (0.25–1.65)	0.96 (0.65–1.44)

(*Continued*)

TABLE I. (*Continued*)

	Genotypes, n				
MTRR 66A-G	**AA**	**AG**	**GG**	**Recessive OR (95% CI)**	**Dominant OR (95% CI)**
Control	41	99	44	Reference group	Reference group
OSD	21	35	21	1.19 (0.65–2.19)	0.76 (0.42–1.41)
SB aperta	23	53	37	1.55 (0.92–2.60)	1.12 (0.63–1.99)
Anencephaly	14	18	12	1.19 (0.57–2.51)	0.61 (0.30–1.27)
Open NTDs	37	71	49	1.44 (0.89–2.33)	0.93 (0.56–1.54)
Total NTDs	64	116	83	1.47 (0.96–2.25)[e]	0.89 (0.57–1.39)

	Genotypes, n				
RFCI 80A-G	**AA**	**AG**	**GG**	**Recessive OR (95% CI)**	**Dominant OR (95% CI)**
Control	35	112	45	Reference group	Reference group
OSD	26	51	26	1.10 (0.63–1.92)	0.66 (0.37–1.17)
SB aperta	21	66	39	1.46 (0.88–2.42)	1.11 (0.61–2.02)
Anencephaly	9	25	15	1.44 (0.72–2.88)	1.00 (0.45–2.27)
Open NTDs	30	91	54	1.46 (0.92–2.32)	1.08 (0.63–1.84)
Total NTDs	62	164	81	1.17 (0.77–1.78)	0.88 (0.56–1.40)

[a] $P = 0.033$.
[b] $P = 0.038$.
[c] $P = 0.03$.
[d] $P = 0.046$.
[e] $P = 0.048$.

detect significant association or the ability to better characterize patients by phenotype.

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