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Report

Maternal Genetic Effects, Exerted by Genes Involved in Homocysteine Remethylation, Influence the Risk of Spina Bifida

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There is currently considerable interest in the relationship between variation in genes that are involved in the folate-homocysteine metabolic axis and the risk of spina bifida. The evaluation of this relationship is, however, complicated by the potential involvement of both the maternal and the embryonic genotype in determination of disease risk. The present study was designed to address questions regarding both maternal and embryonic genetic risk factors for spina bifida by use of the two-step transmission/disequilibrium test. Analysis of data on variants of two genes involved in homocysteine remethylation/methionine biosynthesis—methionine synthase (MTR) A2756G and methionine synthase reductase (MTRR) A66G—provided evidence that both variants influence the risk of spina bifida via the maternal rather than the embryonic genotype. For both variants, the risk of having a child with spina bifida appears to increase with the number of high-risk alleles in the maternal genotype: MTR (R_1 = 2.16, 95% CI 0.92–5.06; R_2 = 6.58, 95% CI 0.87–49.67) and MTRR (R_1 = 2.05, 95% CI 1.05–3.99; R_2 = 3.15, 95% CI 0.92–10.85). These findings highlight the importance of considering both the maternal and embryonic genotype when evaluating putative spina bifida susceptibility loci.

Maternal genetic effects occur when a genetically mediated maternal phenotype influences the phenotype of offspring. Such effects are independent of the alleles contributed by the mother to her offspring and are, therefore, genetic only with respect to the mother. With respect to offspring, maternal genetic effects behave as environmental risk factors. Studies in animals indicate that maternal genetic effects can contribute to the risk of specific malformations among offspring (see, e.g., Trasler and Trasler 1984; Gilliam et al. 1997). The teratogenic effect of untreated maternal phenylketonuria (MIM 261600) provides a classic example of a maternal genetic effect that influences human development (Mabry et al. 1963).

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Research indicating that the risk for neural tube defects (NTD [MIM 601634, MIM 601635]) may be related to functional variants of genes involved in the folate-homocysteine metabolic axis has spurred interest in maternal genetic effects, because such variants could influence the risk of NTD through the maternal, the embryonic, or both the maternal and embryonic genotypes. Case-control studies comparing both subjects with NTD and their mothers to control subjects have provided evidence that the maternal genotypes for 5,10 methylenetetrahydrofolate reductase (MTHFR [MIM 607093]) and methionine synthase reductase (MTRR [MIM 602568]) are associated with the risk of NTD (Whitehead et al. 1995; van der Put et al. 1996; Christensen et al. 1999; Wilson et al. 1999). However, on the basis of the data available from such studies, it is not possible to determine whether these associations reflect the transmission of maternal alleles that exert an effect via the embryonic genotype, maternal genetic effects, or a combination of both maternal and embryonic effects (Posey et al. 1996).

Two family-based approaches for assessing the relationship between disease risk and both maternal and offspring genotypes have recently been proposed (Mitch-

ell 1997; Weinberg et al. 1998; Wilcox et al. 1998). The two-step transmission-disequilibrium test (TDT) (Mitchell 1997) uses data from family trios consisting of a proband and both parents (proband trios) and from trios consisting of the mothers of probands and both maternal grandparents (mother trios). The relationship between proband genotype and risk of disease is assessed in the usual way (Spielman et al. 1993), by evaluating the transmission of alleles from heterozygous parents to their affected offspring. The relationship between maternal genotype and risk of having an affected child is assessed by evaluating the transmission of alleles from heterozygous maternal grandparents of affected individuals to the mothers of those individuals. Therefore, when the proband's genotype is assessed, "being affected with an NTD" is the phenotype of interest, whereas, when the maternal genotype is assessed, "having a child with an NTD" is the phenotype on which the TDT is performed. Both steps in the two-step TDT provide tests of linkage in the presence of linkage disequilibrium and are unbiased by population subdivision or admixture (Ewens and Spielman 1995).

An alternative approach applies log-linear modeling to data derived from only proband trios (Weinberg et al. 1998; Wilcox et al. 1998). This approach characterizes each proband trio according to the number of high-risk alleles (0, 1, or 2) in the genotypes of the father, mother, and child. Asymmetry in the distribution of the proband genotypes, conditional on parental genotypes, permits estimation of the offspring genotypic effects. The likelihood-ratio test (LRT) comparing models with and without parameters characterizing the offspring genotypic effects is a generalization of the TDT. Consequently, this LRT provides a test of linkage in the presence of linkage disequilibrium and is unbiased by population structure or admixture (Weinberg et al. 1998). Estimation of maternal genotypic effects is based on asymmetry in the distribution of reciprocal mating types. The LRT comparing models with and without parameters characterizing the maternal genotypic effects provides a test of association only and is, therefore, subject to bias resulting from population structure or admixture (Wilcox et al. 1998).

The present study was designed to address questions regarding both maternal and offspring genetic risk factors for spina bifida by use of the two-step TDT. In this paper, we describe the results of our analyses of data for variants in two genes involved in homocysteine remethylation/methionine biosynthesis: methionine synthase (MTR [MIM 156570]), A2756G (D919G), and MTRR A66G (I22M). A potential role for these genes in the etiology of spina bifida [MIM 182940] is suggested by the metabolic profile (i.e., relatively elevated homocysteine and relatively low vitamin B12 and red blood cell folate levels [Mills et al. 1995; Lucock et al. 1998, 2000]) of women who have had a child or pregnancy affected with an NTD. Neither embryonic (Morrison et al. 1997; Christensen et al. 1999; Shaw et al. 1999; Johanning et al. 2000) nor maternal (Morrison et al. 1997; Christensen et al. 1999) genetic effects on NTD risk have been demonstrated for the MTR A2576G variant. However, in a small study, MTRR A66G genotypes appeared to be associated with spina bifida in both probands and their mothers (Wilson et al. 1999).

As part of an ongoing study of the genetics of spina bifida (i.e., a defect of the caudal neural tube), families ($n = 209$) that included at least one affected (i.e., with meningocele, meningomyelocele, or myelocele) member were ascertained through several sources: the Spina Bifida Program ($n = 113$) and the Center for Fetal Diagnosis and Therapy ($n = 35$) at the Children's Hospital of Philadelphia; the Spinal Dysfunction Clinic at the Alfred I. duPont Hospital for Children, Wilmington ($n = 51$); advertisements in professional and support group newsletters ($n = 9$); and self referral ($n = 1$). Blood and/or buccal samples were obtained from the family member(s) affected with spina bifida (i.e., the proband) and his or her (their) parents, sibs, and maternal grandparents. The study protocol was approved by the Institutional Review Boards of The University of Pennsylvania School of Medicine and each of the participating hospitals. Informed consent—and assent, when appropriate—were obtained for all study subjects.

Genomic DNA was extracted from whole blood, using GENERATION Capture Columns (Gentra Systems), or from buccal samples, using a published protocol (Walker et al. 1999). The MTR A2756G and MTRR A66G genotyping was performed using heteroduplex generators described elsewhere (Barbaux et al. 2000; Gaughan et al. 2001). Genotypes were read independently by two individuals and were interpreted blind to the identity of the samples. Repeat genotyping assays were performed on all samples that gave inadequate product or equivocal genotypes.

Associations between maternal and offspring MTR and MTRR genotypes and spina bifida were assessed using the two-step TDT (Mitchell 1997). Since the TDT is invalid when data from unambiguous parent-child dyads are included and data from ambiguous dyads are excluded from analysis (Curtis and Sham 1995), only data from complete trios were included in these analyses. Families that included more than one individual with spina bifida contributed one trio for each affected individual, since the TDT remains a valid test of linkage when there is more than one affected individual per family (Spielman and Ewens 1996). These analyses were carried out using the TDT-STDT program (version 1.1).

Associations between maternal and offspring MTR and MTRR genotypes and spina bifida were also assessed using a log-linear approach. To assess the effects

Table 1

Summary of the MTR and MTRR Data and Results Obtained Using the TDT and LRT

	MTR A2756G		MTRR A66G	
VARIANT	Proband Trios	Mother Trios	Proband Trios	Mother Trios
No. of complete trios (no. with one heterozygous parent, no. with two heterozygous parents)	84 (37, 9)	22 (7, 4)	117 (67, 29)	41 (26, 6)
No. of parent-child dyads (F-C, M-C)	48 (5, 43)	36 (12, 24)	42 (3, 39)	27 (6, 21)
No. of informative transmissions[a]	55	15	125	38
No. (%) of informative transmissions of designated high-risk allele	30 (54.6)	13 (86.7)	58 (46.4)	25 (65.8)
TDT:χ^2_1 (P value)	0.46 (0.50)	8.07 (0.004)	0.65 (0.42)	3.79 (0.05)
R_1 (95% CI)[b]	1.18 (0.69–1.98)	2.16 (0.92–5.06)	0.88 (0.56–1.39)	2.05 (1.05–3.99)
R_2 (95% CI)[b]	1.10 (0.34–3.50)	6.58 (0.87–49.67)	0.70 (0.36–1.36)	3.15 (0.92–10.85)
Generalized LRT:χ^2_2 (P value)	0.33 (0.85)	4.59 (0.10)	1.09 (0.58)	5.18 (0.08)
Linearized LRT: χ^2_1 (P value)[c]	…	4.69 (0.03)	…	5.02 (0.02)

[a] Informative transmissions = transmissions from heterozygous parents who are members of a complete trio.
[b] Estimates of R_1 (R_2) and 95% CIs were obtained from the generalized LRT.
[c] The linearized LRT was a post hoc analysis that was applied only to data from mother trios.

of offspring genotype, each proband trio was characterized according to the number of high-risk alleles in the genotypes of the father (F), mother (M), and child (C), and the following log-linear model was fitted to the resulting count data:

$$\ln [E(n_{F,M,C})] = \gamma_j + \beta_1 I_{(C=1)} + \beta_2 I_{(C=2)} + \ln (w_{F,M,C}) \ .$$

The γ_i parameter serves to stratify on mating type. $I_{(C=1)}$ and $I_{(C=2)}$ are dummy variables that take on the value of 1 when C = 1 and C = 2, respectively, and are 0 otherwise. The $w_{F,M,C}$ are cell weights that are: 2 for trios consisting entirely of heterozygotes, and 1 for all other possible combinations. The risks of spina bifida associated with offspring genotypes including one (R_1) or two (R_2) copies of the high-risk allele, relative to the offspring genotype with no copies, were estimated by $\exp(\beta_1)$ and $\exp(\beta_2)$, respectively. The significance of the offspring genotype was assessed using the 2-df LRT comparing the above model to the null model in which $\beta_1 = \beta_2 = 0$. The significance of the maternal genotype and estimates of maternal genotypic relative risks were obtained in an analogous manner, using data from the mother trios.

These log-linear analyses were carried out using LEM (Vermunt 1997), a program for log-linear analysis with missing data that uses the expectation-maximization (EM) algorithm, as described by van den Oord and Vermunt (2000). Using the EM algorithm allows incomplete trios to contribute their information to the LRT without invalidation of the analysis, and has been shown to recapture much of the loss in information from incomplete trios (Weinberg 1999; van den Oord and Vermunt 2000). Hence, data from complete trios (F-M-C) and parent-child dyads (F-C, M-C) were included in these log-linear analyses. In the families that included more than one individual with spina bifida who was available for genotyping, one individual was randomly selected (i.e., coin flip) for inclusion in the proband trio. It is necessary to restrict the analyses in this way to obtain unbiased standard error estimates.

The study sample comprises 209 families that include at least one individual affected with spina bifida. Four of these families include an affected sib pair (n = 213 affected). The results from the two-step TDT and log-linear analyses are summarized in table 1. Briefly, MTR A2756G genotypes were obtained for 84 proband trios, which included 55 heterozygous parents and, hence, 55 transmissions that were informative for the TDT. The informative transmissions included 30 (54.6%) 2756G alleles and 25 (45.4%) 2756A alleles. The difference in the transmission frequency of these alleles, from heterozygous parents to their affected offspring, was not statistically significant.

MTR A2756G genotypes were obtained for 22 mother trios, which included 15 heterozygous maternal grandparents of a child with spina bifida. The informative transmissions included 13 (86.7%) 2756G alleles and only 2 (13.3%) 2756A alleles. The difference in the frequency of transmitted alleles, from heterozygous maternal grandparents to the mothers of a child with spina bifida, was statistically significant, suggesting that the 2756G allele should be designated as the high-risk variant.

MTRR A66G genotypes were obtained for 117 complete proband trios, which included 125 heterozygous parents. The informative transmissions included 58 (46.4%) 66A alleles and 67 (53.6%) 66G alleles. The difference in the transmission of these alleles, from heterozygous parents to their affected offspring, was not statistically significant.

MTRR A66G genotypes were obtained for 41 complete mother trios, which included 38 heterozygous maternal grandparents. The informative transmissions included 25 (65.8%) 66A alleles and 13 (34.2%) 66G alleles. The difference in the transmission of alleles, from heterozygous maternal grandparents to the mothers of a child with spina bifida, was of borderline statistical significance, suggesting that the 66A allele should be designated as the high-risk variant.

Estimates of the relative increase in risk associated with offspring genotypes having one (R_1) or two (R_2) copies of the designated high-risk allele (relative to genotypes with zero copies) were obtained by fitting the full log-linear model to data from all proband trios and dyads. Likewise, estimates of the relative increase in risk associated with maternal genotypes having one (R_1) or two (R_2) copies of the designated high-risk allele were obtained by fitting the full log-linear model to data from all mother trios and dyads.

The relative risk estimates for offspring MTR genotypes were nonsignificant, and the LRT, comparing the fit of the full model to the null model in which β_1 and β_2 (i.e., the parameters defining the contribution of offspring genotype to risk) were set to zero, was also not significant (table 1). In contrast, the relative risk estimates for maternal MTR genotypes suggested that the risk of having a child with spina bifida increases with the number of high-risk alleles in the maternal genotype. However, neither R_1 nor R_2 were statistically significant, and the LRT comparing the fit of the full model to the null model was also not significant.

The relative risk estimates for offspring MTRR genotypes were also nonsignificant as was the LRT comparing the fit of the full model to the model in which β_1 and β_2 were set to zero. In contrast, there was evidence for an increase in risk of spina bifida in the offspring of women with the MTRR AG and AA genotypes, relative to the GG homozygote. The difference in fit between the full and null model was of borderline statistical significance.

For both MTR and MTRR, the estimates of R_1 and R_2 obtained from the mother trios and dyads suggest that the presence of at least one high-risk allele is associated with an increased risk of spina bifida in offspring and that this risk may be further increased when the mother is homozygous for the high-risk allele. Simulation studies have shown that, under such a model, the TDT may outperform the LRT (Weinberg et al. 1998). This difference in power may, at least in part, account for the differences in the level of significance associated with the TDT and LRT for maternal genetic effects. A linearized LRT, which constrains $R_2 = R_1^2$ and is asymptotically equivalent to the TDT (Weinberg et al. 1998), provided stronger evidence than the generalized LRT for maternal genetic effects at the MTR and MTRR loci (table 1).

A recent editorial asked the question, "Why are the genes that cause risk of human neural tube defects so hard to find?" (Harris 2001). The answer to this question, at least in part, may be that we are not looking for these genes in the correct place. The results of the present analyses provide evidence that some of the genes involved in homocysteine remethylation/methionine biosynthesis are likely to influence the risk of spina bifida via maternal rather than embryonic genetic effects. Studies which do not specifically address the potential role of maternal genetic effects on the risk of spina bifida would overlook such effects or, as in case-control studies, would be unable to ascribe definitively the association between offspring phenotype and maternal genotype to maternal, embryonic, or both maternal and embryonic genetic effects.

This study had limitations. The probands in this study may not be representative of all embryos that develop spina bifida. For example, our results could be biased (e.g., an affect could have been missed) if embryos that are homozygous for a high-risk allele are both more likely to develop spina bifida and less likely to be liveborn. The study was also limited by the number of trios available for study. Modest associations between proband genotype and spina bifida may have been missed. The data were also insufficient for the purposes of subgroup analyses and for the assessment of interactions.

The primary strength of this study was its unique design, which provided data for the direct assessment of both maternal and offspring genetic effects on spina bifida risk. This report establishes the feasibility and utility of collecting mother trios and provides the first direct evidence that maternal genetic effects influence the risk of spina bifida. These findings highlight the need to consider maternal genetic effects in the current paradigm of disease etiology for spina bifida and the importance of designing future studies in a manner that will allow for the direct assessment of maternal genetic effects.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Online Medelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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