

Folate-Related Genes and Omphalocele

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Women who take folic acid in the periconceptional period greatly reduce their chances of having a child with a neural tube defect (NTD). Using multivitamins may also reduce the risk of having a child with an omphalocele. In this study, we tested single nucleotide polymorphisms in folate-related enzyme genes for association with omphalocele. Polymorphisms in methylenetetrahydrofolate reductase (*MTHFR*), methylenetetrahydrofolate dehydrogenase (*MTHFD1*), the reduced folate carrier (*SLC19A1*), and transcobalamin II (*TCN2*) were examined in 25 children with euploid omphalocele and 59 matched controls. Omphalocele cases were significantly more likely to carry the T allele of *MTHFR* 677C → T, a known risk factor for NTDs (odds ratio 3.50, 95% confidence interval 1.07–11.47, *P* = 0.035). The *MTHFD1* R653Q, *SLC19A1* R27H, and *TCN2* P259R polymorphisms showed no significant association with omphalocele. In this small study, the thermolabile variant of *MTHFR*, 677C → T, was associated with an increased risk for omphalocele. This variant causes reduced enzyme activity, thus suggesting a mechanism by which multivitamins with folic acid might prevent omphalocele. Additional investigation is required.

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INTRODUCTION

It is well known that folic acid, taken periconceptionally, can prevent neural tube defects (NTDs) [MRC, 1991; Czeizel and Dudas, 1992] and there is considerable evidence that folate-related genes play an important part in susceptibility to NTDs. In particular, a variant in methyltetrahydrofolate homocysteine methyltransferase (commonly known as methylenetetrahydrofolate reductase), *MTHFR* 677 C→T (A222V) has been shown to be a risk factor for NTDs [van der Put et al., 1995; Whitehead et al., 1995; Botto and Yang, 2000]. We have recently

reported that mothers who have the trifunctional enzyme—methylenetetrahydrofolate dehydrogenase (5,10 methylenetetrahydrofolate dehydrogenase, 5,10 methylenetetrahydrofolate cyclohydrolase, 10 formyltetrahydrofolate synthetase) variant *MTHFD1* R653Q are at significantly increased risk of having children with NTDs [Brody et al., 2002]. Other gene variants including those for transcobalamin II (*TCN2*) and the reduced folate carrier (*SLC19A1*, also known as *RFC1*) have also been associated with increased risk of NTDs [De Marco et al., 2003; Pietrzyk and Bik-Multanowski, 2003] in association with low vitamin B12 levels [Morin et al., 2003], but not in all studies [Afman et al., 2002; Shaw et al., 2002].

Omphalocele is a defect of the anterior abdominal wall that ranges in birth prevalence from 0.8 to 3.9 per 10,000 births [Stoll et al., 2001]. Many cases have chromosomal abnormalities or syndromes, but the etiology of the remaining cases is largely unknown.

Women who use multivitamins, most of which contain folic acid, have been reported to be at reduced risk for having children with omphalocele [Botto et al., 2002]. We hypothesized that folate-related genes may be important in the etiology of omphalocele as well as NTDs. Because non-aneuploid omphalocele cases are rare and it is difficult to find large numbers for study, we present the results of a pilot study addressing this hypothesis.

MATERIALS AND METHODS

Omphalocele was defined as a midline abdominal wall defect limited to an open umbilical ring. The viscera herniate into the base of the umbilical cord and are covered by an amnioperitoneal membrane.

Subjects for this study were selected from all births in New York State for the years 1998 and 1999. Omphalocele cases for this investigation came from the New York State Congenital Malformations Registry (NYSCMR), one of the largest state-wide, population-based birth defects registries in the United States [New York State Department of Health-Congenital Malformations Registry Annual Report]. Cases are ascertained passively via reports from physicians and hospitals. Reporting of major congenital malformations diagnosed from birth through 2 years of age is required by law. For each defect, the reporting physician or hospital is asked to provide a narrative describing the defect. Malformation rates obtained for New York State are similar to those obtained by systems that use active case finding [Salihu et al., 2003]. To help ensure completeness of reporting, a second data source is used. NYSCMR cases are matched to hospital discharge data also filed with the New York State Department of Health. Details on any cases on the NYSCMR list that the hospital did not report are requested directly from the hospital. A recent capture-recapture analysis showed that the NYSCMR was about 87% complete [Honein and Paulozzi, 1999]. A similar estimate of 89% was recently obtained after matching Congenital Malformations Registry reports (New York State Department of

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Health) obtained for a regional study using active case-ascertainment. Data on cases that are terminated after omphaloceles are discovered by prenatal diagnosis are not available; however, a research study showed that 20% of cases (5/25) in New York would be missed by using only birth and stillbirth records. We did not try to estimate how many trisomic omphalocele cases were born in our population because the diagnosis of omphalocele is sometimes omitted in trisomy cases, and our protocol called for these cases to be excluded anyway.

Using data provided by the NYSCMR, residual newborn screening specimens for cases were taken from storage and the samples were assigned random numbers. All identifying information was then removed. Potential control samples were also taken from storage, assigned random numbers, and de-identified.

Cases and controls were matched by gender and by race/ethnicity—Non-Hispanic White, African-American, Hispanic, Asian, and other—as reported to the newborn screening data base by the hospital of birth. Controls were obtained by taking all births without major malformations and stratifying them by race. Random numbers were then used to select controls of the same race and gender as each case. In order to run the entire set of samples in a single assay, the number of controls was limited to 59. Each case subject had at least two and no more than three matched control samples included in the analysis. All samples were stripped of identifying information prior to shipping to the genotyping laboratory.

Ethical approval was obtained for this study from the NIH Office of Human Subjects Research and New York State Department of Health Institutional Review Board.

Genetic Testing

DNA purification. Samples for genetic analysis were obtained from filter paper collected by the New York State Newborn Screening Program. Genomic DNA was purified from dried blood spots taken from newborn screening (Guthrie) cards. A single 3 mm circle was punched from each card directly into 96-well reaction plates. Cases and matched controls were randomly assigned to wells within the plate. DNA was purified using Genra Generation Capture Card reagents essentially as directed by the manufacturer except a second DNA elution step was added. Two microliters of this second elution were used for each genotyping assay.

Genotyping. A single SNP was studied per gene. SNPs were selected because they result in an amino acid change known to affect gene function and/or are a change associated with increased risk for other birth defects. DNA including the polymorphic sites was PCR amplified and used as a template for a primer extension reaction. Allele-specific extension products were detected and scored by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Sequenom). Primer sequences and assay conditions are available upon request.

Statistical analysis. Cases and controls were compared using a stratified analysis based on the Mantel–Haenszel test for two-by-two tables [Breslow and Day, 1980]. For analysis, two different models were considered: a dominant model, in which the effects of the homozygous and heterozygous genotypes are assumed to be identical, and a recessive model, in which only the homozygous genotype is assumed to have an effect. In either case, the data can be collapsed to a two-by-two table. For analysis the samples were stratified by race/ethnicity. This analysis assumes a common odds ratio in each stratum, although the underlying rates are allowed to differ. This approach was chosen because of the small number of cases within each racial/ethnic group. The Mantel–Haenszel test performs well with fairly small samples, as long as the number

of strata is small as is the case here. In addition, an extension of the “rule of five” for the expected frequencies in a two-by-two table was used to check the adequacy of the single-degree-of-freedom chi-square approximation for the Mantel–Haenszel statistic [Breslow and Day, 1980]. According to this rule, the expected number of cases at risk across strata is calculated under the null hypothesis; this is the expected frequency used in the chi-square statistic. If this value differs by at least five from the maximum and minimum possible values of the total number of exposed cases, then the chi-square approximation is considered adequate. In addition the Breslow–Day test [Breslow and Day, 1980] was used to check the homogeneity of the odds-ratio. Nominal P values < 0.05 were considered significant. As this was a hypothesis generating study, correction for multiple comparisons was not carried out.

RESULTS

After exclusion of cases with chromosomal abnormalities, there were 37 omphalocele cases without known chromosomal abnormalities identified among the 546,050 births in New York in 1998–1999. Of these, 25 had samples available for DNA testing; 12 did not because the samples had been used up in the neonatal screening process. Nineteen cases had isolated omphalocele; six had other defects: one had cloacal exstrophy, one had a cardiac malformation, two had renal defects, and two had Beckwith–Wiedemann syndrome.

There were 10 male and 15 female cases. The ethnic distribution was 12 Non-Hispanic White (48%), 7 Non-Hispanic Black (28%), 0 Asian (0%), 5 Hispanic (20%), and 1 other (4%). For all births over the same time period and geographical area, there was a slightly higher proportion of Non-Hispanic Whites (53.4%) and Asians (6.7%), and a lower proportion of Non-Hispanic Blacks (18.8%). The proportion of Hispanics was almost identical (20.5%). As expected because of the matching, the racial distribution of the cases and controls did not differ ($P=1.0$), nor did the proportion of female cases (60%) compared to female controls (61%) $P=0.9$. The mean age of the case mothers (27.8 years.) did not differ significantly ($P=0.5$) from the mean age of the control mothers (28.8 years.). As the samples were anonymized prior to testing, information on socioeconomic status and other demographic characteristics was not available. There were 59 controls matched as described above to the cases.

To determine whether data from different ethnic groups could be examined together, the Breslow–Day test for homogeneity of odds ratios was applied. Odds ratios were homogeneous for all except the test for a dominant association between omphalocele and *TCN2*. Therefore, data for the different ethnic/racial groups are presented together in Table I.

There was a significant association between the T allele (dominant test) of *MTHFR* 677 and omphalocele, adjusted odds ratio 3.50, 95% confidence interval 1.07–11.47, $P=0.035$. See Table I. The extended rule of five indicated that the chi-square approximation was adequate (see Materials and Methods). The test for a recessive association was not significant; however, there were only six subjects (one case and five controls) with the TT genotype. When we repeated the analysis excluding the two Beckwith–Wiedemann cases, the association between the T allele (dominant test) and omphalocele had an odds ratio of 2.90, 95% confidence interval 0.89–9.85, $P=0.07$.

The *MTHFD1*, *TCN2*, and *SLC19A1* polymorphisms showed no significant association with omphalocele, either as dominant or recessive effects (Table I). The *TCN2* polymorphism did not have homogeneous odds ratios by race/ethnicity (the Breslow–Day test for homogeneity was statistically significant for a test of dominant effect) so the data were examined for each race separately. There was no significant association between this polymorphism and omphalocele for any race/ethnicity.

TABLE I. Polymorphism Rates in Omphalocele Cases and Control Subjects^aN(%)

<i>MTHFR</i>	CC	CT	TT		OR ^b	95% CI
677C→T						
Omphalocele	11 (44)	13 (52)	1 (4)	T dominant $P = 0.035$	3.50	(1.07–11.47)
Control	38 (64)	16 (27)	5 (8)	T recessive $P = 0.62$	0.57	(0.06–5.38)
<i>SLC19A1</i>						
R27H	AA	AG	GG			
Omphalocele	7 (28)	11 (44)	7 (28)	A dominant $P = 0.87$	1.10	(0.35–3.44)
Control	13 (22)	29 (50)	16 (28)	A recessive $P = 0.45$	1.57	(0.50–4.93)
<i>TCN2</i>						
P259R	CC	GC	GG			
Omphalocele	6 (25)	13 (54)	5 (21)	G dominant $P = *$	*	*
Control	9 (33)	28 (49)	10 (18)	G recessive $P = 0.60$	1.37	(0.41–4.52)
<i>MTHFD1</i>						
R653Q	AA	GA	GG			
Omphalocele	3 (12)	14 (56)	8 (32)	A dominant $P = 0.74$	1.19	(0.43–3.27)
Control	9 (16)	28 (48)	21 (36)	A recessive $P = 0.66$	0.74	(0.19–2.94)

^aMissing data *SLC19A1*=1, *TCN2*=3, *MTHFD1*=1.

^bExcludes three “other race” subjects.

*Odds ratio not homogeneous by Breslow–Day.

There was no evidence of an interaction between *MTHFR* and *SLC19A1* ($P = 0.77$).

DISCUSSION

To the best of our knowledge, this is the first study to look for an association between folate related genes and omphalocele. Studying the etiology of omphalocele is difficult because so many cases are aneuploid and must be excluded. Despite this limitation, we were able to demonstrate that the *MTHFR* CT plus TT genotypes were significantly more common in omphalocele cases than in controls. *MTHFR* is an important gene in folate metabolism, providing methyl groups for the conversion of homocysteine to methionine, and subsequent methylation reactions. The *MTHFR* 677 C→T variant has reduced biological activity. The *MTHFR* 677 C→T polymorphism is also a well known risk factor for NTDs [van der Put et al., 1995; Whitehead et al., 1995; Botto and Yang, 2000] and periconceptional vitamin use greatly reduces the risk of NTDs. Thus, our finding that *MTHFR* is associated with omphalocele provides biological support for, and a potential mechanism to explain, the observation that women who take multivitamins are at lower risk for having a child with an omphalocele [Botto et al., 2002].

Most studies of *MTHFR* 677 C→T and NTD have reported an association with the homozygous variant (TT). In contrast, we found that omphalocele is associated with the combined CT and TT group. We have shown recently [Kirke et al., 2004] that the CT genotype is also a statistically significant risk factor for NTDs. Relton et al., 2004 have recently reported that the T allele is a risk factor as well. The meta-analysis by Botto and Yang [2000] also showed that the CT heterozygote was a risk factor for NTDs, but the association did not quite reach statistical significance. The most likely reason that we did not see a TT effect is that we had very few subjects with the TT genotype in this study, limiting our power to find an association for TT alone.

Some limitations of our study should be noted. This is a small study with limited power to rule out associations. Thus, we would not claim that our data totally exclude the possibility that the variants we tested in *TCN2*, *SLC19A1*, or *MTHFD1* could be related to omphalocele. Although we selected SNPs that are known to alter function, we emphasize that other variants in these genes that were not examined could be risk factors. Because the birth prevalence of omphalocele is low, we pooled genotype data from our cases from different racial/ethnic groups. Because allele frequencies differ by racial/ethnic group, population stratification may be a confounder.

To reduce the risk of population stratification, all cases were matched to controls on race/ethnicity [Wacholder et al., 2002]. The method used to classify race/ethnicity was the same for cases and controls, and was independent of diagnosis. Such a study design greatly reduces the likelihood of stratification artifacts. Moreover, the Mantel–Haenszel test was stratified by race, and the analysis included a test for racial heterogeneity. Data on maternal use of folic acid supplements and dietary folate consumption would have been useful to determine how the risk of omphalocele was affected by folic acid exposure. Collecting such data prospectively on the half million plus pregnancies from which our cases and controls were identified, however, was impossible and ethical constraints prevented us from interviewing the control subjects to gather the data retrospectively. Maternal DNA could not be collected, making it impossible to test for maternal risk factors. Lastly, we tested a number of hypotheses, so positive findings could have occurred by chance. The fact that *MTHFR* 677 C→T is an established risk factor for birth defects makes this less likely. Our statistical analysis was very conservative; all studies show that the T allele only increases the risk for birth defects. Our statistical testing was two-sided, that is, assuming that the T allele could also prevent birth defects. If we use a one-sided test, our P values for the association between the T allele (dominant test) and omphalocele become $P = 0.018$ for the entire cohort and $P = 0.035$ when Beckwith–Wiedemann cases are excluded. We would encourage others to follow up on our findings on the relationship between omphalocele and these folate-related genes.

In summary, this study found that *MTHFR* 677 CT and TT genotypes were significantly more common in children with omphalocele than in normal control children. *MTHFR* 677 C→T causes reduced function in vivo leading to lower red cell folate and higher homocysteine levels. Thus, our finding that *MTHFR* 677 C→T is associated with omphalocele supports the observation that folic acid containing multivitamins may prevent omphalocele.

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