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Association of Folate Receptor (*FOLR1*, *FOLR2*, *FOLR3*) and Reduced Folate Carrier (*SLC19A1*) Genes with Meningomyelocele

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Abstract

BACKGROUND—Meningomyelocele (MM) results from lack of closure of the neural tube during embryologic development. Periconceptional folic acid supplementation is a modifier of MM risk in humans, leading to an interest in the folate transport genes as potential candidates for association to MM.

METHODS—This study used the SNPlex Genotyping (ABI, Foster City, CA) platform to genotype 20 single polymorphic variants across the folate receptor genes (*FOLR1*, *FOLR2*, *FOLR3*) and the folate carrier gene (*SLC19A1*) to assess their association to MM. The study population included 329 trio and 281 duo families. Only cases with MM were included. Genetic association was assessed using the transmission disequilibrium test in PLINK.

RESULTS—A variant in the *FOLR2* gene (rs13908), three linked variants in the *FOLR3* gene (rs7925545, rs7926875, rs7926987), and two variants in the *SLC19A1* gene (rs1888530 and rs3788200) were statistically significant for association to MM in our population.

CONCLUSION—This study involved the analyses of selected single nucleotide polymorphisms across the folate receptor genes and the folate carrier gene in a large population sample. It provided evidence that the rare alleles of specific single nucleotide polymorphisms within these genes appear to be statistically significant for association to MM in the patient population that was tested.

Keywords

neural tube; meningomyelocele; folic acid; folate transport genes; single nucleotide polymorphisms

INTRODUCTION

Neural tube defects (NTDs) are common malformations of the brain and spinal cord, and they include all abnormalities resulting from lack of closure of the developing neural tube during embryologic development. The causes of human neural tube defects are largely unknown, but are almost certainly multifactorial, consisting of both genetic and environmental components (Finnell et al., 2000; Cabrera et al., 2004; Detrait et al., 2005; Beaudin and Stover 2009). No single major gene has been implicated in the etiology of these disorders across all populations studied to date. It seems likely that NTDs involve multiple variants across a single gene or variant effects across a number of genes. Furthermore, the variants and genes may differ between ethnic groups. Previously, definitive results have been hampered by the lack of reproducibility of the association studies implicating certain genes (Hirschhorn et al., 2002; Beaudin and Stover 2009; Greene et al., 2009; Copp and Greene 2010). Linkage studies have yielded some interesting results (Rampersaud et al., 2005). In addition, animal models have proved to be a useful tool to gain some insight into the molecular processes of neural tube formation and closure. In clinical severity, NTDs range from mild forms (e.g., spina bifida occulta) that often do not require surgical intervention to lethal forms such as anencephaly (Botto et al., 1999).

Meningomyelocele (MM) is the most severe form of spina bifida that is compatible with survival. In an MM, both the meninges and the spinal cord protrude through a gap in the vertebral column, and the lesion is not covered by the skin. Although these defects can occur at any point along the developing neural tube, lumbosacral lesions are the most common (Hunter et al., 1996). As a result of current surgical and medical interventions, most children born with MM in the United States survive, although in the first year of life the mortality risk of these infants is greater than in the general population. Despite these interventions, children born with an MM almost invariably have profound, life-long disabilities (Detrait et al., 2005).

It is known that maternal folate status is a modifier of NTD risk (Botto et al., 2005). These findings make the folate metabolic pathway genes potential candidate genes for association to meningomyelocele. The folate transport genes are involved in the transport and maintenance of intracellular levels of folate. FOLR1 and FOLR2 are glycosyl phosphatidylinositol (GPI)-anchored proteins that facilitate unidirectional transport of folates (Verma et al., 1992; Henderson et al., 1995), whereas FOLR3 codes for a secreted form of the receptor protein (Shen et al., 1995). SLC19A1 is a cell surface transmembrane protein that participates in bidirectional movement of folate across the membrane (Matherly et al., 2007; Hou and Matherly, 2009). Because of the role that these proteins play in the maintenance of critical levels of intracellular folate, it is logical to hypothesize that functional variants within these genes are associated with MM. In fact, it has been found that polymorphisms in the *SLC19A1* gene, such as the A80G variant, appear to confer susceptibility to MM risk in some populations (Shang et al., 2008; Pei et al., 2009). Furthermore, the knockout mouse models of the *FOLR1* and *SLC19A1* genes result in embryonic lethality. Failure of neural tube closure is one of the abnormal morphologic findings in the null embryos (Peidrahita et al., 1999; Gelineau-van Waes et al., 2008). This finding provides more compelling evidence that these genes play a role in neural tube closure and thereby potentially in MM risk.

The FOLR1 receptors are expressed on the microvillus plasma membrane of the placenta where, in combination with the proton-coupled high affinity folate transporters (PCFT), appear to be responsible for the internalization and cytoplasmic release of folate (Solanky et al., 2010). In contrast, the reduced folate carrier (SLC19A1), a bidirectional transporter of

primarily reduced folates, is expressed on both the microvillus plasma membrane and the basolateral plasma membrane of the placenta (Solanky et al., 2010).

The present study focused on the folate transporter genes as candidates for association to MM. The rationale for choosing these genes was based on epidemiologic studies, genetic association studies by other groups, biologic function of candidate genes, and animal models (MRC Vitamin Study Research Group, 1991; Czeizel and Dudas, 1992; Piedrahita et al., 1999; Williams et al., 2002; De Marco et al., 2003; Zhu et al., 2007; Shang et al., 2008; Pei et al., 2009).

MATERIALS AND METHODS

Study Population

The majority of the MM cohort tested in the study consists of Caucasians of European descent and Hispanics of Mexican descent in the United States (Table 1). The MM probands and their parents were enrolled after obtaining informed consent. The patient cohort was recruited primarily from five different sites: Houston, Texas; the Texas-Mexico border area; Lexington, Kentucky; Los Angeles, California; and Toronto, Ontario, Canada. Recruitment took place during clinical visits, hospitalizations, or at parent meetings. The probands were born between 1955 and 2008 (Au et al., 2008), and the ages at enrollment ranged from 6 months to adulthood. The criteria for inclusion were based on whether an individual had an MM or was related to an affected individual. The exclusion criteria were the presence of spina bifida that was not associated with an MM or a syndromic form of spina bifida. No individual was excluded on the basis of race or sex. In the study, 329 affected child-parent trios and 281 affected child-parent duos were tested. The level of defect was determined by the review of medical records and also, in the case of some of the affected individuals, by the review of radiographs. Maternal health history, pregnancy history, maternal exposures, and sociodemographic information were obtained from the parents of the affected children. Information about vitamin supplementation was not obtained, but a study (food frequency questionnaire) is now underway that includes this information. Because family association studies, specifically transmission disequilibrium test (TDT) analyses, were used as the primary statistical tool, the family trios (consisting of the father, mother, and affected child) were the most important component of the population for the study. The project was approved by the Institutional Review Board of the University of Texas Health Science Center at Houston.

A total of 92 anonymous Hispanic subjects with no personal or family history of NTDs were enrolled in the Houston area to obtain appropriate Hispanic control sample frequencies for the tested single nucleotide polymorphisms (SNPs). To obtain SNP frequencies for Caucasian Americans, a Caucasian control DNA panel (HD100CAU) was purchased from the Coriell Institute (Camden, NJ). DNA samples from 30 Caucasian families used in the HapMap project (<http://www.hapmap.org>) were included in the genotyping as a quality control measure for genotype calls.

DNA Genotyping

A blood sample was obtained from the proband and both parents when available. Genomic DNA was extracted from the pelleted white blood cells using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN). The Oragene DNA Preparation Kit (DNA Genotek, Ontario, Canada) was used to prepare DNA from saliva samples when a blood sample could not be obtained.

DNA samples for 610 affected members and 939 parents of the MM population, 90 HD100Cau, and 92 Hispanic controls were genotyped for the selected SNPs. DNA samples

from 82 individuals from 15 CEU families were also included. The genotypes of many of these individuals are available in the HapMap project, providing an internal quality control for the genotyping assay.

The testing interrogated SNPs in the folate transport genes. As mentioned previously, these genes are integral players in the delivery and transport of folate and are therefore considered to be good candidate genes for affecting MM formation. In the design of the SNPset, both tag and non-tag SNPs were included to increase coverage (Table 2). The folate receptor genes *FOLR1*, *FOLR2*, and *FOLR3* map to human chromosome 11q13.4, and the genomic DNA sizes are 6.74 kb, 5.15 kb, and 4.16 kb, respectively. The reduced folate carrier *SLC19A1* gene is located on 21q22.3 and is 27.72 kb in size. When possible, SNPs with a minimum heterozygosity of 5%, as reported in the HapMap CEU population, were chosen. The databases that were used for SNPset design were: <http://www.ncbi.nlm.nih.gov>, <http://genome.ucsc.edu>, <http://www.hapmap.org>, <http://www.genecards.org>, and <http://snp.wustl.edu>.

The genotyping platform used was the SNPlex Genotyping System (Applied Biosystems, Foster City, CA). Based on the SNP sequences submitted, Applied Biosystems designed allele SNP-specific probes for each SNP. The SNPs were submitted as standard SNP Identifiers (SNP IDs) from the common available databases. Based on compatibility of probes representing each SNP, ABI assembled the most compatible SNPs into a SNPset. The SNPlex platform uses the ligation of these allele-specific probes for each SNP that hybridized to the patient genomic DNA SNP loci, followed by multiplex PCR amplification. The design strategy enables allelic discrimination of SNPs at specific positions in the human genome (Martinez et al., 2009; Shaw et al., 2009). A 5- μ l (200 ng) aliquot of genomic DNA was used per reaction, and the standard SNPlex genotyping protocol was used. The subsequent electrophoresis of the SNP probes was performed on the ABI 3730xl DNA analyzer, and genotype calls were made using GeneMapper v4.0.

Data Analyses

Our SNP selection approach yielded 48 potential SNPs, but only 20 SNPs across the four folate transport genes (*FOLR1*, *FOLR2*, *FOLR3*, *SLC19A1*) met all the criteria to be submitted for statistical analyses (Table 2). Sixteen of the 28 excluded SNPs had clustering errors, four had Mendelian inconsistencies, six were of Hardy Weinberg Equilibrium (HWE), and two did not reach the acceptable ($\geq 90\%$) genotype concordance call rate with the internal CEU (U.S. residents with northern and western European ancestry collected by the Centre d'Etude du Polymorphisme Humain) controls when the data were available on HapMap (<http://hapmap.ncbi.nlm.nih.gov/>)

To minimize the effect of missing genotypes on the subsequent association study, only the SNPs that reached an acceptable genotype call rate of $\geq 90\%$ were considered for statistical analyses. Prior to association analyses, all family units were tested for the presence of Mendelian errors. To control for genotype call error, an SNP was re-examined if 10 families or more (i.e., $>10/610$ or $>1.64\%$) displayed Mendelian errors. If an SNP did not pass on the first quality control test, it was re-examined to make a second genotype call for an additional quality control test. If it again failed to meet the quality control criteria, it was removed from the association study. HWE was determined based on 237 controls by ethnicity. Therefore, different ethnic groups were not pooled, and it could be determined that the successful SNPs did not deviate from HWE ($p < 0.05$). The final data analyses involved family-based studies of genetic association, notably the TDT using the TDT component of the PLINK v1.07 (<http://pngu.mgh.harvard.edu/purcell/plink/>) whole genome association analysis toolset (Purcell et al., 2007). Our MM patient cohort consists of a number of duo (family unit consisting of either the mother and affected child or the father and affected child). In these

cases, the unit is usually missing the father (Martinez et al., 2009). To increase the power in the study, it is important to use as many of the cases as possible. The PLINK algorithm uses the duo families and allows for the missing parents. Furthermore, this analytical method generates counts for transmitted and nontransmitted alleles.

RESULTS

The allele frequencies of the 20 SNPs that passed all quality control criteria are listed in Table 3. A total of 10 SNPs demonstrated significant association with MM risk in this study (Table 4).

One SNP in the *FOLR1* gene showed significant association with MM. The *FOLR1* SNP rs35179028 is a synonymous coding SNP (p.V132V) with a low minor allele frequency (Table 3). The positive result, therefore, has to be interpreted with caution (Table 4).

Two SNPs (rs35982790) and (rs13908) in *FOLR2* showed significant association with MM. The intronic SNP rs35982790 has a low minor allele frequency (Table 3), and again the association has to be interpreted with caution (Table 4).

The three significant SNPs—notably rs7925545, rs7926987, and rs7926875 in the *FOLR3* gene—are tightly linked in the CEU population (<http://www.HapMap.org>) (The International HapMap Consortium, 2007) and have TDT *p* values of 3.85E^{-04} , 1.96E^{-02} , 1.84E^{-02} respectively (Table 4). The rs7925545 SNP lies in the 5' region close to the gene, whereas the rs7926987 and rs7926875 variants lie within intron 2. The *FOLR3* synonymous coding SNPs rs508088 and rs34970007 are significant, but both SNPs have low minor allele frequencies (Table 3).

Finally, two SNPs (rs1888530 and rs3788200) in *SLC19A1* showed significant association with MM. The rs3788200 is located in intron 2 (TDT *p* value, 1.95E^{-02}). The rs1888530 variant that was significantly associated with MM in the study is located in intron 5 of the *SLC19A1* gene (TDT *p* value, 7.28E^{-05} ; Table 4).

DISCUSSION

In our study, a number of SNP variants in the folate receptor genes and the folate carrier gene showed preferential transmission from parent to offspring and are associated with MM in the population tested.

The folate receptor 2 gene (*FOLR2*) is located on chromosome 11 and is 5.2 kb in size; it consists of five exons and four introns. The variant found in the gene (rs13908) is a nonsynonymous SNP (A → G) located in exon 2 and is the most interesting SNP studied, because of potential functional significance. The variant results in a missense mutation with the lysine (AAG) at this position substituted for a glutamic acid (GAG). The Lys at position 35 is conserved in a multiple species alignment (<http://genome.ucsc.edu/>) (Kuhn et al., 2007). The amino acid is also conserved among all three folate receptors (FOLR1, FOLR2, FOLR3), again indicating potential functional significance (Fig. 1). The conserved Lys is at amino acid position 35 in the FOLR2 protein and is found as part of a conserved motif consisting of [Lys]-[His]-[His]-[Lys]. The function of this motif is currently unknown, but FOLR2 is a GPI-anchored protein that binds, internalizes, and unloads 5-methyltetrahydrofolate and other folate derivatives to the interior of the cell. The receptor then has to recycle back to the cell surface for an additional round of ligand binding. The variant is not located in the GPI site (Yan and Ratnam, 1995); however, if the motif is involved in any other critical processes and the amino acid substitution (Lys→Glu) disrupts the process, it could potentially lead to decreased levels of intracellular folate during critical

periods of embryonic development. It would be useful to further validate the association by replicating the finding in an independent MM population.

The FOLR1 and FOLR2 receptors are membrane-associated, GPI-anchored receptors (Lacey et al., 1989; Yan and Ratnam, 1995). The FOLR3 receptor, in contrast, is a constitutively secreted form of the folate receptor (Shen et al., 1995). Little has been published about the FOLR3 protein, and there is limited information regarding the role of the FOLR3 receptor in folate transport. The gene is located on chromosome 11 and is approximately 4.2 kb in size. It consists of five exons and four introns. Three SNPs within the *FOLR3* gene (rs7925545, rs7926987, and rs7926875), showed statistically significant TDT values. In addition, each of the SNPs had an acceptable genotype concordance call rate with the internal CEU controls (91%, 93%, and 91%, respectively). All four SNPs are in tight linkage disequilibrium in the CEU population (www.HapMap.org); therefore, they would be expected to segregate together and to behave in a similar fashion (i.e., show similar association patterns). The D' and r^2 values were calculated using the CEU data (www.Hap-Map.org) and Haploview v 4.1 (<http://www.broadinstitute.org/haploview/haploview>).

None of the SNPs in *FOLR3* have been evaluated previously for MM risk, and currently no putative function can be assigned. The SNPs rs7926875 and rs7926987 are located in intron 2 of the *FOLR3* gene. The rs7925545 SNP is located in the intergenic region approximately 1230 bp upstream from exon 1 of the gene and is conserved among primates (<http://genome.ucsc.edu/>). Two alternative promoter regions have been suggested for the *FOLR3* gene, with both located <500 bp upstream from exon 1 of the gene (<http://www.ncbi.nlm.nih.gov/>) (The National Center for Biotechnology Information). The rs7925545 SNP may be located within the upstream promoter-enhancer region. The *FOLR3* SNPs rs7926987 and rs7926875 are conserved among primates (<http://genome.ucsc.edu/>).

The reduced folate carrier gene *SLC19A1* is located on chromosome 21, and it is approximately 28 kb in size. The gene consists of six exons and five introns. Six SNPs in this gene were analyzed for association. However, only the rs3788200 located in intron 2 and the rs1888530 SNP located in intron 5 were found to be significantly associated with MM. Although neither of these SNPs have been previously evaluated for MM risk, a nonsynonymous variant in exon 2 (rs1051266) has shown association with NTD risk in three other studies of two population groups (Shang et al., 2008; Franke et al., 2009; Pei et al., 2009). It is important to note that the mouse knockout for the *SLC19A1* gene is embryonic lethal. The phenotype can be partially rescued when the pregnant dams receive folic acid supplementation (Gelineau-van Waes et al., 2008).

A major strength of our study is the large population size (Table 1). In addition, the use of family-based association studies, specifically the TDT, as an analytical tool is a strength in addition to the availability of a sizable number of trio families (Table 1) facilitates the use of this approach. The TDT statistical method uses the nontransmitted parental alleles as controls, thereby effectively addressing issues such as population structure, which can be problematic in case versus control analyses. Approximately 60% of the study population is Hispanic of Mexican descent. Hispanics are of particular interest because, in the United States, Hispanics of Mexican descent have a higher risk than any other ethnic group of having a child affected with an NTD (Canfield et al., 1996; Canfield et al., 2009).

Our study involved a SNP screen across the three folate receptor genes (*FOLR1*, *FOLR2*, *FOLR3*) and the reduced folate carrier gene (*SLC19A1*) in a large population sample consisting of approximately 60% Hispanics of Mexican descent. A number of SNPs across these genes were found to be associated with a protective effect for MM in the population

tested. Furthermore, this study is the first to associate the *FOLR3* gene with MM. It would be useful to validate our findings in a second, independently ascertained MM population. If the same variants are again found to be associated with MM in a second population, then functional studies should be designed for these SNPs in an attempt to determine their biologic roles.

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Figure 1 shows an amino acid sequence alignment of three human folate receptors: FOLR1, FOLR2, and FOLR3. The alignment is presented in a standard format with the protein names and their corresponding amino acid sequences. The residue p. Lys35Glu (rs13908) of FOLR2 is highlighted in a shaded box to indicate conservation.

Figure 1.
Amino acid sequence alignment between the three human folate receptors. Note: The location of p. Lys35Glu (rs13908) of FOLR2 is shaded to show conservation.

Table 1**Study Population**

Characteristic	Trios	Duos	Total
Race			
Caucasian	142	84	226
Mexican American	168	166	334
African American	4	14	18
Asian American	2	2	4
Other ^a	7	5	12
Unknown ^b	6	10	16
Total	329	281	610
Sex			
Male	154	126	280
Female	167	144	311
Unknown	8	11	19
MM Lesion Level			
≥L1	81	81	162
≤L2	203	169	372
Mixed	5	4	9
Unknown	40	27	67

^a Native American and other country of origin.

^b Unknown ethnicity.

Table 2
SNPs Tested across the Four Folate Transport Genes (*FOLR1*, *FOLR2*, *FOLR3*, *SLC19A1*)

Gene Symbol	Gene Name	Gene Size (kb)	Chromosome	SNP	Chromosomal Location	Significance
<i>FOLR1</i>	Folate Receptor 1	6.74	11	rs2071010	71,578,612	Intron/utr Isoform 3
				rs3833748	71,579,110	Intron
				rs35179028	71,584,342	Val132Val
<i>FOLR2</i>	Folate Receptor 2	5.15	11	rs651933	71,604,308	5' near gene
				rs35982790	71,606,664	Intron
				rs13908	71,607,379	Lys35Glu
				rs514933	71,607,855	Intron
<i>FOLR3</i>	Folate Receptor 3	4.16	11	rs7925055	71,522,744	5' near gene
				rs7925545	71,523,189	5' near gene
				rs7926875	71,527,090	Intron
				rs7926987	71,527,151	Intron
				rs508088	71,528,304	Ala173Ala
<i>SLC19A1</i>	Solute carrier family 19 member 1	27.72	21	rs34970007	71,528,379	Lys198Lys
				rs11235449	71,528,847	3' near gene
				rs1888530	45,760,851	Intron
				rs12482346	45,762,055	Intron
				rs2838958	45,772,995	Intron
				rs9282854	45,775,894	Leu262Leu
				rs914232	45,777,178	Intron
				rs3788200	45,780,999	Intron

SNP, Single nucleotide polymorphism.

Table 3

Single Nucleotide Polymorphism Allele Frequencies for the Folate Receptor and Carrier Genes (Based on 237 Controls) by Ethnicity

Gene Symbol	Marker	Allele 1	Allele 2	Caucasian (n = 145)	Hispanic (n = 92)
<i>FOLR1</i>	rs2071010	A	G	0.05/0.95	0.10/0.90
	rs3833748	delG	G	0.00/1.00	0.05/0.95
	rs35179028	A	G	mono	0.01/0.99
<i>FOLR2</i>	rs651933	A	G	0.47/0.53	0.36/0.64
	rs35982790	C	A	1.00/0.00	0.96/0.04
	rs13908	A	G	0.90/0.10	0.59/0.41
	rs514933	A	G	0.57/0.43	0.74/0.26
<i>FOLR3</i>	rs7925055	A	G	0.96/0.04	0.94/0.06
	rs7925545	A	G	0.98/0.02	0.98/0.02
	rs7926875	A	C	0.05/0.95	0.05/0.95
	rs7926987	C	G	0.95/0.05	0.93/0.07
	rs508088	C	T	1.00/0.00	0.95/0.05
<i>SLC19A1</i>	rs34970007	A	G	0.02/0.98	0.04/0.96
	rs11235449	A	G	0.43/0.57	0.22/0.78
	rs1888530	C	T	0.15/0.85	0.11/0.89
	rs12482346	C	T	0.61/0.39	0.55/0.45
	rs2838958	A	G	0.58/0.42	0.47/0.53
	rs9282854	G	A	0.70/0.30	0.72/0.28
	rs914232	C	T	0.75/0.25	0.60/0.40
	rs3788200	A	G	0.38/0.62	0.34/0.66

Table 4

Family-Based Association on Total Population for FOLR1, FOLR2, FOLR3, and SLC19A1 Transmission/Disequilibrium Test Analysis ($p < 0.05$) Performed Using PLINK

GENE	CHR	SNP	A1 ¹	A2 ²	T ³	U ⁴	OR	χ^2 value	p value
<i>FOLR1</i>	11	rs35179028	A	G	7	19	0.3684	5.538	0.0186
<i>FOLR2</i>	11	rs35982790	C	A	3	21	0.1429	13.5	0.0002386
<i>FOLR2</i>	11	rs13908	G	A	39	62	0.629	5.238	0.0221
<i>FOLR3</i>	11	rs7925545	G	A	7	28	0.25	12.6	0.0003857
<i>FOLR3</i>	11	rs7926875	A	C	4	14	0.2857	5.556	0.01842
<i>FOLR3</i>	11	rs7926987	G	C	11	25	0.44	5.444	0.01963
<i>FOLR3</i>	11	rs508088	T	C	2	15	0.1333	9.941	0.001616
<i>FOLR3</i>	11	rs34970007	A	G	5	19	0.2632	8.167	0.004267
<i>SLC19A</i>	21	rs1888530	C	T	25	62	0.4032	15.74	0.00007284
<i>SLC19A</i>	21	rs3788200	A	G	37	60	0.6167	5.454	0.01953

CHR, chromosome; SNP, single nucleotide polymorphism; A1¹, common allele; A1², minor allele; T³, number of cases with minor allele transmitted; U⁴, number of cases with minor allele not transmitted; OR, odds ratio.