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Mutations in Folate Transporter Genes and Risk for Human Myelomeningocele

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Abstract

The molecular mechanisms linking folate deficiency and neural tube defect (NTD) risk in offspring remain unclear. Folate transporters (SLC19A1, SLC46A1, SLC25A32, and FOLH1) and folate receptors (FOLR1, FOLR2, and FOLR3) are suggested to play essential roles in transporting folate from maternal intestinal lumen to the developing embryo. Loss of function variants in these genes may affect folate availability and contribute to NTD risk. This study examines whether variants within the folate transporter and receptor genes are associated with an increased risk for myelomeningocele (MM).

Exons and their flanking intron sequences of 348 MM subjects were sequenced using the Sanger sequencing method and/or next generation sequencing to identify variants. Frequencies of alleles of single nucleotide polymorphisms (SNPs) in MM subjects were compared to those from ethnically-matched reference populations to evaluate alleles' associated risk for MM. We identified eight novel variants in *SLC19A1* and twelve novel variants in *FOLR1, FOLR2*, and *FOLR3*. Pathogenic variants include c.1265delG in *SLC19A1* resulting in an early stop codon, four large insertion deletion variants in *FOLR3*, and a stop_gain variant in *FOLR3*. No new variants were identified in *SLC46A1, SLC25A32, or FOLH1.* In *SLC19A1*, c.80A>G (rs1051266) was not associated with our MM cohort; we did observe a variant allele G frequency of 61.7%, higher than previously reported in other NTD populations. In conclusion, we discovered novel loss of function variants in genes involved in folate transport in MM subjects. Our results support the growing evidence of associations between genes involved in folate transport and susceptibility to NTDs.

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Folate transport mutations

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Keywords

myelomeningocele (MM); neural tube defects (NTDs); folate transporters; folate receptors

INTRODUCTION

Neural tube defects (NTDs) are congenital anomalies that occur when the neural tube fails to close completely early in fetal development. The worldwide prevalence of NTDs is 1 to 10 per 1,000 births, making it the second most common congenital birth defect after congenital heart defects [Au et al., 2010]. Anencephaly, the most severe form of NTD, is the result of a defect occurring at the rostral end of the neural tube, leading to incomplete formation of the brain and skull and subsequently death soon after birth. In contrast, spina bifida arises when the defect occurs at the level of the vertebral column, resulting in impaired neural function caudal to the level of the defect. Myelomeningocele (MM), the most common type of spina bifida, is characterized by an open defect with herniation of the spinal cord or nerves [Detrait et al., 2005; Copp et al., 2010]. Exposure of the spinal nerves to amniotic fluid during in-utero development leads to degeneration and loss of neural tissue, resulting in life-long disabilities and reduced life expectancy [Bowman et al., 2001; Talamonti et al., 2007; Oakeshott et al., 2010; Adzick 2012].

It is well established in the literature that both genetic and environmental factors contribute to NTD risk [Cabrera et al., 2004; Agopian et al., 2013]. In the case of environmental influences, folic acid (FA) fortification of cereal and grain products has been widely successful in reducing the incidence of NTD associated with nutritional deficiency [Boulet et al., 2008; Mosley et al., 2008]. After the FDA implementation of mandatory FA fortification of grain products in 1998, the incidence of spina bifida in the United States declined by 35.5% and anencephaly by 25.9%, further validating folate deficiency as a risk factor for NTD formation [Williams et al., 2015]. Some animal models also demonstrate reduction of genetic NTD risk with periconceptional folate supplementation [Gelineau-van Waes et al., 2008; Copp et al., 2010]. However, the biologic relationship between folate and NTDs is unclear. No single gene has been etiologically implicated as causative of NTDs, thus association studies have targeted candidate genes in the folate metabolic pathway [Martinez et al., 2009; Marini et al., 2011; Etheredge et al., 2012; Zhang et al., 2013].

We postulate that folate transporter and receptor genes have critical roles in supplying folate/FA from the pregnant mother through the placenta to maintain normal development of the neural tube during embryogenesis (Figure 1). Folate consumed by the mother is absorbed in the small intestine, enters the maternal circulation, and is preferentially distributed to the placenta to be delivered and taken up by the developing embryo through a series of folate transporters and receptors [Yasuda et al., 2008; Solansky et al., 2010]. Our group has previously demonstrated the association of folate transport genes with MM using a family-based transmission disequilibrium test [O'Byrne et al., 2010]. The genes of interest in our study are *SLC19A1* (also known as reduced folate carrier or *RFC1*), *SLC46A1* (also known as proton-coupled folate transporter or *PCFT*), *SLC25A32* (also known as mitochondrial folate transporter or *MFTC*), *FOLH1* (also known as folate hydrolase), and

folate receptor genes *FOLR1, FOLR2*, and *FOLR3.* Once consumed by the pregnant mother, maternal FOLH1 converts the dietary form of folate, polyglutamate-folate, into monoglutamate-folate to be transported across the apical brush-border membrane of the small intestine via folate transporters SLC46A1 and SLC19A1 [DeVos et al., 2008; O'Byrne et al., 2010; Zhao et al., 2013]. Folate is stored in the liver in the form of 5-methyltetrahydrofolate (5-MTHF) and released into the maternal circulation [Zhao et al., 2011]. Circulating 5-MTHF binds to folate receptors (e.g. FOLR1) that lie on the maternal side of the placenta. It has been theorized that FOLR1 and SLC46A1 are responsible for the transport of 5-MTHF via acidified endosomes during receptor-mediated endocytosis into the cytoplasm of the syncytiotrophoblast where it is then transported across the basement membrane by SLC19A1 [Solanksy et al., 2010]. Once in the fetal circulation, 5-MTHF is transported into cells via folate receptors. FOLR1 and FOLR2 are glycosylphosphatidylinositol (GPI)-anchored proteins with high affinity for folate and transport folate via endocytosis [Verma et al., 1992; Sabharanjak et al., 2004; Solanky et al., 2010]. FOLR3, in contrast, lacks a glycosylphosphatidylinositol (GPI) signal and is a constitutively-secreted form of folate receptor [Shen et al., 1995]. Ubiquitous in cells and tissues, SLC19A1 is also responsible for transporting reduced folate into the cytoplasm, while the mitochondrial folate transporter, SLC25A32, transports cytoplasmic folate across the inner mitochondrial membrane to take part in the mitochondrial folate metabolism cycle [Titus et al., 2000].

Previous genetic epidemiology studies have investigated genes of folate one-carbon metabolism and methionine cycle, but less attention has been paid to folate transport genes with the exception of *SLC19A1* [Au et al., 2016 AJMG same issue]. Knockout mouse models of *Slc19a1* rescued with folinic acid supplementation, a folic acid derivative, exhibit NTDs, supporting the role of *SLC19A1* in NTD formation; however human NTD populations have demonstrated variable degrees of association with the folate transporters [Gelineau-van Waes et al., 2008; Wang et al., 2012]. Only one study recently investigated *SLC46A1* in cases of NTD or cleft lip and palate and failed to show an association [VanderMeer et al., 2016]. Clinical presentations of loss of function mutations of *SLC46A1* have been reported in cases of hereditary folate malabsorption, consistent with nullizygous mouse models of *Slc46a1* [Salojin et al., 2011; Zhao et al., 2011; Shin et al., 2012; Diop-Bove et al., 2013]. *SLC25A32* nullizygous mouse embryos exhibited 100% penetrance of neural tube defects, but it is unknown if the defects were compatible with life [Kim, 2016]. In the human population, a SNP in *SLC25A32* was associated with lower plasma folate levels in a Japanese population, but this gene has not been explicitly studied in human NTDs [Urano et al., 2014]. Earlier association studies involving *FOLH1* and NTDs have been conflicting, but it has been suggested that maternal *FOLH1* polymorphisms may increase NTD risk by influencing folate and homocysteine levels [Guo et al., 2013]. *Folh1*$^{-/-}$ mouse model do not present with obvious birth defects but morphologically demonstrate significantly reduced axon area of the sciatic nerve suggesting a possible role in myelination of developing axons [Bacich et al., 2005].

In regards to folate receptors, *Folr1* knockout mice rescued with folate present with anterior NTDs along with cardiovascular anomalies [Zhu et al., 2007]. Human NTD cohorts have shown indirect evidence of the function folate receptors play during pregnancy with reports

of low serum folic acid receptor α levels and increased levels of folate receptor autoantibodies found in mothers of NTD patients [Yang et al., 2016]. However, no association of *FOLR1* or *FOLR2* was discovered in earlier NTD cohorts until our group first reported an association by family based transmission-disequilibrium test in 2010 by O'Byrne et al. [Barber et al., 1998; Heil et al., 1999; O'Leary et al., 2003; Boyles et al., 2006]. In the same article, we also were the first to investigate and report association between *FOLR3* and MM, but the protein function of *FOLR3* in NTDs is unknown.

Thus it is our aim to examine S*LC46A1, SLC19A1, SLC25A32, FOLH1, FOLR1, FOLR2*, and *FOLR3*, for genetic variants in a large cohort of MM subjects to establish a more complete picture of the molecular mechanisms in folate deficiency and NTD risk. We hypothesize that variants within the folate transporter and receptor genes are associated with MM risk.

MATERIALS AND METHODS

Subjects were selected from a cohort of nonsyndromic MM subjects enrolled in ongoing genetic studies in our laboratory [Au et al., 2008]. These subjects were enrolled after obtaining informed consent between 1996 and 2006 from spina bifida clinics at three sites (Houston, Texas; Los Angeles, California; and Toronto, Canada). The research was approved by the Institutional Review Board at The University of Texas Health Science Center at Houston. The study sample selected for Sanger sequencing comprised of 96 MM affected subjects who were born before 1998, pre-FA fortification era, to include MM phenotypes that may not present after FA fortification to improve our chances of identifying genetic variations as previously described [Aneji et al., 2012; Tilley et al., 2012]. Additionally, another 252 MM subjects were sequenced by whole exome sequencing (WES) from the same cohort of MM affected subjects previously described [Au et al., 2008]. These subjects were born before and after 1998, up through 2008.

DNA extraction

Genomic DNA was extracted from blood lymphocytes using the Puregene DNA extraction kid (Gentra Systems Inc, Minneapolis, MN). Parental saliva samples were collected in some cases when blood samples were not available and the saliva DNA was prepped using the Oragene DNA preparation kid (DNA Genotek; Kanata, Ontario, Canada) following manufacturer's protocols. Working DNA was prepared as previously described [Ruggiero et al., 2015].

Polymerase Chain Reaction Amplification

Polymerase chain reaction (PCR) and nested-sequencing primers were designed for each gene of interest based on the GRCh37 reference genomic sequences recorded in the University of Santa Cruz Genome Browser (UCSC Genome Browser; http://genome.uscs.edu/cgi-bin/hgGateway). The primers were designed containing approximately 100 bases flanking the exons to include splice donor and acceptor sites. In light of the presence of pseudogenes, special attention was paid to selecting primers with the least homology to the corresponding pseudogenes (i.e. *FOLR1P1* and *FOLR3P1*). Primers were

synthesized by Integrated DNA Technologies USA (Commercial Park, Coralville, IA). The exons were amplified by hot-start-PCR with MyTaq-HS DNA Polymerase (Bioline USA Inc, Tuanton, MA) using the MJ Research PTC-100 Thermal Cycler (MJ Research, Waltham, MA). Gel electrophoresis verified the expected sizes of PCR products. The amplified products were treated with exonuclease I and rapid alkaline phosphatase (United States Biochemicals, Affymetrix, Cleveland, OH) to remove excess primers and nucleotides before sequencing.

Sequencing

Sanger sequencing was conducted using the BigDye Terminator Protocol (LifeTechnologies Inc., Foster City, CA) with nested-sequencing primers. An alternative protocol was used with GC enhancer buffer (LifeTechnologies Inc) to amplify high guanine-cytosine region. The sequencing products were resolved on the ABI3130 Genetic Analyzer (Life Technologies Inc., Grand Island, NY).

Whole exome sequencing (WES) using the Ion Proton System was performed following the standard workflow of the manufacturer (Thermo Fisher Scientific). Sequencing templates between 100-400 bp were prepared from an aliquot of 1.0 ug of subject genomic DNAs using Ion Xpress™ Plus Fragment Library Kit with the AB Library Builder™ System. In the process, probes consisting of enhanced-exome region were used to capture and enrich subject genomic DNA templates matching the enhanced-exome region. The captured subject DNA sequencing templates were applied to the Ion PI Chip v3 for sequencing using the Ion Proton Sequencer. Sequencing results were analyzed using the Ion Torrent Suite Software v4.4 and mapped to reference human genome sequence (GRCh37/hg19), and sequences that differed from GRCh37/hg19 were called variants. Variants were annotated with reference to the latest released version of variants recorded by Single Nucleotide Polymorphism Database (dbSNP 144).

Analysis of Sequencing Data

For Sanger sequencing, we anticipated that a sample size of 96 subjects to examine 192 chromosomes would allow detection of rare variant allele with a frequency ≥0.52% (1/192). Sanger sequencing results were manually compared with the reference sequence (RefSeq) for each of the folate transporting genes to identify SNPs and novel variants. Each sequencing result was examined by at least two different members of the research team. Variants not previously reported in dbSNP 144 were considered novel and confirmed by sequencing from both directions using PCR product from a fresh preparation of genomic DNA. When available, parental genomic DNA of subjects with novel variants were sequenced from both directions to determine if the variants were *de novo* or inherited.

Ethnically-matched reference populations were used for comparison against identified SNPs. The Caucasian reference population is derived from (1) the 1KGenomes project (www.ncbi.nlm.nih.gov/variation/tools/1000genomes) and (2) the NHLBI Exome Sequencing Project (evs.gs.washington.edu/EVS). The Mexican-American reference population is from (1) the 1KGenomes Project and (2) collaborators at The University of

Texas School of Public Health at Houston. There are no known reports of NTDs in the reference populations.

RESULTS

Novel Variants Discovered in the Study

Solute Carrier Family—We identified a total of eight novel variants in *SLC19A1* using the Sanger sequencing method and WES (Table I). One notable novel pathogenic variant involves a single base deletion (c.1268delG) in exon 5 of *SLC19A1*. The single base deletion causes a shift of the translation reading frame (p.G423Afs*12) leading to a premature stop codon 12 amino acids downstream resulting in loss of translation of the last transmembrane domain and the cytoplasmic domain (Figure 2). This region is crucial for stability of SLC19A1 as loss of the cytoplasmic domain is known to completely inactivate the protein [Sharina et al., 2002]. Seven additional novel variants in introns across *SLC19A1* were identified by WES in the 252 MM subjects (Table I). Functional significance of these variants is not known. No novel missense variants, novel stop_gain/loss or novel variants affecting splice sites were identified in *SLC46A1* nor in *SLC25A32* in the 252 MM subjects tested by WES.

Folate Receptor Family—Among the folate receptor genes, we discovered one novel variant in *FOLR1*, four novel variants in *FOLR2*, and nine novel variants in *FOLR3* (Table I). Among them, five novel variants in *FOLR3* involving deletion/insertion/duplication are predicted to be damaging which we will describe here. Two variants involve deletion of exon 2 and the splice donor that involves the translation initiation codon ATG and 55 amino acids containing the signal peptide (amino acid 1–23) required for docking the ribosome to the endoplasmic reticulum (ER) and translating the protein into the ER lumen. Two other variants involve deletion of the majority of exon 5 together with the splice acceptor. Exon 5 of *FOLR3* codes for 81 amino acids and the translation stop codon TGA. One MM subject had both an exon 2 (c.18_168+18del) and an exon 5 (c.493+39_723del) deletion variant but it is not known whether the two deletion variants were in cis or trans. Deficient or complete loss of functional *FOLR3* mRNA would be expected if both exon 2 and exon 5 deletion variants are in cis-position. Only c.18_168+18del was found in the genome of the subject's mother, suggesting that variant was maternally inherited while c.493+39_723del was *de novo*. The last novel pathogenic variant is a stop_gain c.579G>A (p.W193X) resulting in loss of 50 amino acids in the C-terminus of *FOLR3*.

We discovered several variants in *FOLR2* and *FOLR3* that may impact splicing and transcription, resulting in loss of protein function. One potentially important 173bp duplication consisting of 63bp of the 5′-UTR region in exon 1 of *FOLR2* and 110bp of intron 1 including the splice donor site. The duplicated splice donor site may lead to cryptic splicing altering the optimal secondary structure of the 5′-UTR for the *FOLR2* mRNA and likely affecting mRNA stability and/or translation. The exact functional significance for the two variants needs to be validated. Three novel intronic variants in *FOLR2* and two in *FOLR3* were discovered within 50 bases of the splice site motifs. Analysis using Human Splicing Finder 3.0 (http://www.umd.be/HSF3/HSF.html) suggests the novel intronic

variants alter the sequence motifs of branch point, exonic splicing enhancers, or exonic splicing silencer at the locations and potentially affect splicing of the adjacent exons.

Two novel intronic variants in *FOLR3* (c.-6-40t>c and c.41g>c) were found in seven MM subjects and are located adjacent to each other at positions 71846952 and 71846953 of chromosome 11, 40 and 41 bases upstream respectively from the splice acceptor junction of exon 2 (Table II). The c.-6-40c constitutes a Bsu36I restriction enzyme site. We digested the PCR products of these subjects and sequenced the intact fragment to reveal the c.-6-40t and c.41g are in cis. The two Bsu36I fragments were extracted and re-ligated for PCR sequencing and the results demonstrated the digested fragment consisted of c.-6-40c and c.-6-41c. The c.-6-40t>c and c.41g>c occurred *de novo* in one subject and was inherited from a parent for the remaining six subjects. To examine the potential functional significance of this gt>cc variant, we referred to the Human Splicing Finder online tool, demonstrating a cryptic splice acceptors of almost equal strength upstream the splice acceptor preceding exon 2 [Desmet et al., 2009]. With the gt>cc change, the cryptic splice acceptor immediately following the double allele variants is predicted to increase preference for splice factors binding and splicing. Splicing at the cryptic splice acceptor will add 34 bases to the 5′-UTR of the *FOLR3* mRNA and thus potentially affect ribosomal binding to the mis-spliced mRNA and the mRNA instability. Of further interest, multiple species have the cc allele, however humans are the only species that have the intronic gt sequences allele. From an evolutionary standpoint, cc are the ancestral alleles that can be found in less evolved species than the human such as a chimp and gorilla. The human alleles gt are generally considered to be evolved, and reversion of the human allele gt back to the less evolved ancestral allele cc could be disadvantageous.

A fourth member of the folate receptor family, *FOLR4* (also known as *IZUMO1R* or *JUNO*), shares phylogeny with the other folate receptors but lacks folate-binding capabilities due to several amino acid differences. It plays an essential role in reproduction in mammals as an egg receptor for Izumo1, a sperm-egg fusion protein, and may modulate gene expression in the uterus, but homozygous offspring in mice do not exhibit birth defects [Salbaum JM et al., 2013; Bianchi et al., 2014]. We examined *FOLR4* for completeness in examining all genes in the folate receptor family, and as expected, no novel or vary rare missense, stop_gain/loss or splice site variants were discovered by WES.

SNPs Discovered in the Study

Very Rare SNPs—A total of 21 very rare SNPs in *SLC19A1* were discovered; nine in 96 MM patients by Sanger sequencing and 12 in 252 MM subjects by WES (Table III). The rare allele frequencies for these SNPs shown in dbSNP146 are between 0 and 0.03. No very rare SNPs contributing to missense, stop_gain/loss or affecting splice sites were identified in *SLC46A1* nor *SLC25A32* of the 252 MM subjects.

Four of the 21 very rare SNPs found in *SLC19A1* lead to missense changes including c. 941C>T (rs200236009), and c.971C>T (rs200647386) found by Sanger sequencing in Caucasian MM subjects and c.532G>A (rs760930392) and c.584T>A (rs756426597) found by WES in Hispanic MM subjects. Sequencing of parental genomic DNA determined the father to be a carrier of mutation c.532G>A and the mother to be a carrier of mutation c.

584T>A suggesting they were inherited allelic changes. The remaining SNPs were *de novo* mutations. All four of the very rare missense variants identified were predicted to have some damaging effect by one of the commonly used protein functional analyses algorithms (PROVEAN, SIFT, PolyPhen2 and Mutation Taster). It has been shown that amino acids localized in several transmembrane domains (TMD) including TMD4, TMD5, TMD7, TMD8, TMD10, and TMD11 play important roles in forming the folic acid-binding pocket [Hou et al., 2005, 2006]. For example, c.532G>A results in a replacement of neutral glycine to hydrophilic serine at the end of TMD5 which may affect the stability of TMD5 within the membrane lipid bilayer environment. Likewise, c.584T>A replaces phenylalanine with tyrosine with the addition of a hydrophilic hydroxyl group, potentially destabilizing the hydrophobic environment between TMDs of SLC19A1 needed for folate binding and transportation. Lastly, c.941C>T changes a polar amino acid, threonine, to a nonpolar methionine which may influence folate binding affinity of S313, an amino acid required for the binding of folate [Hou et al., 2006].

Common SNPs—The allele frequencies of common SNPs found in folate transporter genes are shown in Table IV. Two-tailed Fisher's test was performed to compare allele frequencies from MM subjects and the ethnically-matched reference populations. Two SNPs in *SLC19A1* (rs150492570, rs11284347) and two SNPs in *FOLR3* (rs139130389, rs1802608) were found to be associated with MM subjects with nominal significance ($p<0.05$). Approximately six SNPs occurred in MM subjects that have not been seen among European or Mexican populations in 1KGenomes project. Another seven SNPs found in MM subjects were only observed in next generation sequencing combined cohorts EVA-ExAc of 60,706 individuals (aggregated from multiple studies listed http://exac.broadinstitute.org/faq).

DISCUSSION

Folate is necessary for synthesis of DNA and some amino acids as well as for regulation of gene expression. During pregnancy folate demands increase by 5- to 10-fold to meet the needs of the developing fetus [Antony et al., 2007]. As mammals cannot produce folate *de novo*, the supply of folate to the developing embryo is dependent on the dietary intake of the pregnant mother in conjunction with a series of maternal and placental folate transporter proteins. Maintaining an adequate folate supply to the developing fetus relies on normal function of folate transport proteins in the mother, placenta, and embryonic cells. It is plausible that during gestation when folate demands are high, pathogenic variants in folate transporter and receptor genes in the mother and/or fetus could exacerbate a folate-deficient environment or even predispose a mother towards folate deficiency despite adequate folate intake. In our study we evaluated the genetic roles of a series of folate transporters and receptors in MM subjects.

The most studied *SLC19A1* polymorphism in NTD research is A80G (rs1051266) and has been shown to be associated with MM risk in sample populations from Italy, the United States, and China, but not in the United Kingdom [Shaw et al., 2002; De Marco et al., 2003; Relton et al., 2004; O'Leary et al., 2006; Pei et al., 2009]. A meta-analysis of these studies suggests that the A80G polymorphism is not an independent risk factor for NTD [Wang et

al., 2012]. However, it is important to note that folate supplementation was voluntary and unregulated in most of the countries where these studies were conducted. When we combined NTD studies in countries with voluntary food FA fortification policies in place [De Marco, Relton, O'Leary] and compared them to NTD studies in countries pre-FA fortification or without fortification policies [Shaw, Pei, our study] and performed a Chi-square analysis, we found a statistically significant difference in the GG allele frequency (0.301 and 0.365, respectively; p-value 0.0468) (Table V). We would expect a higher A80G occurrence rate in populations without FA fortification. Our finding corroborates the report of *Slc19a1* animal models demonstrating the folate-sensitivity of the protein [Gelineau-van Waes et al., 2008]. In our cohort of 96 MM patients representing a population born during the pre-FA fortification era, the homozygous (GG) allele frequency was 37.5% among Caucasians and 41.7% among Hispanics, among the highest when compared to the above-mentioned studies. We did not show an association between A80G and our MM cohort, however our control population of Mexican Americans derived from the 1K Genomes Project exhibited a GG allele frequency higher than other control populations described in prior association studies involving A80G, which could point towards a genetic risk factor that may predispose U.S.-born Hispanics to myelomeningocele, an ethnic group with the highest rate of NTDs in the U.S. [Canfield et al., 2009].

Folate receptor genes have not been as extensively studied in NTD populations compared to other folate-related genes such as *MTHFR* and *SLC19A1*. While *Folr* knockout NTD animal models exist, previous studies have failed to identify novel variants in *FOLR1* and *FOLR2* in NTD patients but excluded examination of the UTRs and the promoter regions [Barber et al., 1998; Heil et al., 1999]. The function of *FOLR3* in NTD is largely unknown, and knowledge of the biological function of FOLR3 is limited except expression has been correlated to progression of carcinomas [Corrigan et al., 2014]. However, we discovered five novel variants with deleterious consequences in the *FOLR3* gene of four MM subjects strongly supporting variation in *FOLR3* to be a risk factor for MM development. Consistent with our findings in an earlier study, the current study identified multiple novel loss-of-function variants in the folate receptor gene *FOLR3* and noncoding variants with potential functional significance in *FOLR1*, *FOLR2*, and *FOLR3* of MM subjects [O'Byrne et al, 2010]. We propose folate receptors play a bigger role in NTD development than previously recognized.

We find it significant that no variants have been discovered in coding regions of *FOLH1, SLC46A1*, and *SLC25A32* in MM subjects. As neural tube closure occurs prior to embryonic gut development, *FOLH1* and *SLC46A1* expression may not be involved in neural tube development. Further investigation is warranted examining these genes in the mothers of MM subjects as impaired protein enzyme activity could inhibit intestinal absorption during pregnancy further impacting in-utero neural tube development.

Strengths and Limitations

There are several strengths of our study. Our study cohort includes the two ethnicities (Mexican American and Caucasian) that have the highest rates of NTDs in North America. In addition, we were able to study a large sample population totaling 348 MM cases. Lastly,

we used parental DNA of the MM subjects to determine heritability patterns of novel variants.

Some limitations of our study include a lack of data on maternal dietary intake of folate or folate levels. Also, our study used a retrospective design. Moreover, we had a small sample size of subjects for whom we utilized Sanger sequencing and a low minor allele frequency of SNPs thus restricting the power of the study to identify significant associations between SNPs with low rare allele frequency and our study cohort affected with MM. Therefore, due to our overall sample size, caution should be used when comparing the rare allele frequency in our subject population with reported population frequencies. This study is limited to examining the genetic variants effects on folate transportation in relation to the placental and fetal tissues because we only tested affected subjects with full sequencing. Presence of un-transmitted genetic variants in the pregnant mother were not detected as parental samples were only studied to determine status (inherited vs. *de novo*) of variants found in affected subjects. In addition, this study only examined the exomes and non-coding flanking sequences that may affect splicing. Currently, our knowledge and techniques to determine function of non-coding genetic variants remains limited. However, we cannot exclude the possibility for the presence of functional non-coding variants in the folate transportation genes contributing to risk of MM.

In summary, our results reinforce the growing evidence supporting genetic associations of genes involved in folate metabolism pathway and the susceptibility to NTDs. In future directions on research involving folate transporting proteins and NTDs, we are investigating how folate deficiency may directly impact gene expression of folate transporters. We have found expression of *FOLR1* and *SLC19A1* is down regulated along with several other folate cycle related genes in human fibroblasts grown in folate-deficient conditions [Nolan et al., manuscript in prep]. This may have important implications on the gene-nutrient interactions associated with NTD risk. Our current study highlights the function of various folate transporters and receptors beginning at maternal dietary intake to ultimately meet the folate demands of the developing embryo. The low occurrence of pathogenic variants among MM subjects in the study suggests genetic variants in the folate transportation genes play a small role in the development of MM. Further study is needed in mothers of MM subjects to elucidate genetic risk factors in folate transportation genes to evaluate whether variants in of these genes present in the maternal genome may contribute to folate deficiency and increase risk of MM in the offspring. In addition, we need to pursue additional studies assessing the potential impact these newly discovered variants have on the physiologic function of genes in the folate metabolism pathway. Comparing these findings to unaffected controls will further help to improve our understanding of the contribution that both genetic and environmental factors have in the development of NTDs.

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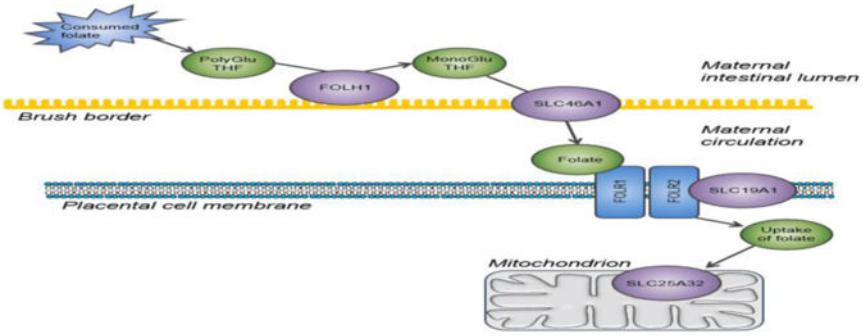

Figure 1.

Folate transport pathway from mother to embryo. Folate refers to natural dietary folates and synthetic folic acid used in fortification and supplements. Folate transporters (FOLH1, SLC46A1, SLC19A1, and SLC25A32) and folate receptors (FOLR1 and FOLR2) are responsible for maternal intestinal absorption and transport of folate into cells including placental cells and the developing embryo to be utilized in metabolic pathways including the methionine cycle, homocysteine metabolic pathway, and folate mediated one-carbon metabolic pathway. Note: THF, tetrahydrofolate; FOLH, folate hydrolase; FOLR, folate receptor; SLC, human solute carrier. *Modified from Petri V et al.*

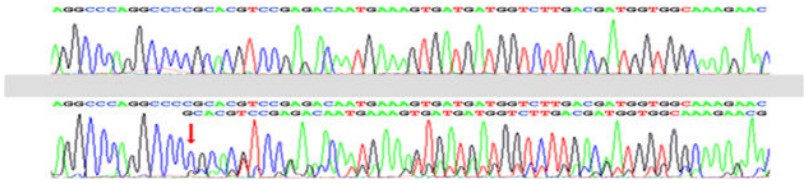

Figure 2.

Deletion of one base (c.1268delG; p.G423Afs*12) in *SLC19A1* identified in a MM subject. Note: A, adenine; T, thymine; G, guanine; C, cytosine. Top panel represents normal reference sequences and sequencing trace of reverse sequences of *SLC19A1* exon 4. Bottom panel shows the corresponding heterozygote sequencing traces consisting of the normal and the variant allele with deletion of a “C” nucleotide. The deletion of “C” shown on the reverse strand of *SLC19A1* exon 4 will be transcribed into c.1268delG in cDNA, resulting in shifting of translation reading frame by one base changing codon 423 from G to A. The frame-shifting mutation in cDNA will lead to premature termination of translation of SLC19A1 (p.G423Afs*12).

Table I

New sequence variants identified among MM subjects.

Gene	cDNA change	Significance	Frequency	GERP	Method
SLC19A1	c.1268delG	p.G423Afs *12	1/338†	4.51	Sanger/WES
SLC19A1	c.1151+98G>C	intron	1/262‡	−4.71	WES
SLC19A1	c.1151+100T>C	intron	1/262‡	0.19	WES
SLC19A1	c.1151+1256N>A	intron	1/262‡	−0.90	WES
SLC19A1	c.1151+1259N>A	intron	1/262‡	0.45	WES
SLC19A1	c.1152−960C>A	intron	1/242†	0.43	WES
SLC19A1	c.1293+334C>T	intron	1/242†	−5.2	WES
SLC19A1	c.1294−4627G>A	intron	1/262‡	−0.62	WES
FOLR1	c.−143C>T	5′-UTR; disrupted mIR target	1/131‡	−1.03	Sanger
FOLR2	c.−87_−25+110dup	5′-UTR/intron duplication	1/87†1	NA	Sanger
FOLR2	c.-24-45C>A	intron, ESE broken	1/343‡	2.44	Sanger/WES
FOLR2	c.339+13_339+14delinsA	intron, new ESE and branch point	1/338†	−0.82	Sanger/WES
FOLR2	c.339+34C>T	intron, new ESE	1/355‡	0.68	Sanger/WES
FOLR3	c.-6-41G>C	intron, ESE broken	6/90‡, 1/95†	1.85	Sanger
FOLR3	c.-6-40T>C	intron	6/90‡, 1/95†	−3.11	Sanger
FOLR3	c.-6-49_168+18delins94	del exon 2, cryptic splicing	1/95†	NA	Sanger
FOLR3	c.16C>T; c.18_168+18del	del exon 2	1/95†§	NA	Sanger
FOLR3	c.168+43G>T	new ESE, ESS broken	1/355‡	−0.02	Sanger/WES
FOLR3	c.493+5_692del	del exons 4–5	1/93‡	NA	Sanger
FOLR3	c.493+39_723del	del exons 4–5	1/93†§	NA	Sanger
FOLR3	c.494−32G>C	ESE broken	1/335†	−0.43	Sanger/WES
FOLR3	c.579G>A	p.W193X; stop gain	1/335†	2.94	Sanger/WES

Note: A1, reference allele; A2, alternative (rare) allele.

[*] Represents stop codon of cDNA,

[†] represents the frequency for Caucasian MM subjects' 2N chromosomes,

[≠] represents frequency for Hispanic MM subjects 2N chromosomes.

[§] Found in the same patient.

[1] Indicates variants identified from the same MM patient approximately 3.3Kbp apart. WES, whole exome sequencing; ESE, exonic splicing enhancer; ESS, exonic splicing silencer. Functional significance of intronic alleles were examined using the online Human Splicing Finger v3.0 (http://www.umd.be/HSF3).

Table II

Change of 11:71846952-53 gt>cc may create a cryptic new splice acceptor site for *FOLR3* exon 2. Sequences of the 11:71846952-53 region was analyzed using the online tool for Human Splicing Finder v2.4.1 (HSFv2.4.1). Although all MM individuals who carry one variant also carry the other variant, we evaluate all possible combinations of the two variants (gt, ct, gc and cc) for potential effects on splicing of the *FOLR3* exon 2. Compare to the reference sequence exon 2 splice acceptor (SA), the HSF results show the 11:71846952-53 gt (c.-6-41g and c.-6-40t) are at the −7 −6 position of a putative splice acceptor site (cagGC) with similar HSF matrics score (87.52 vs 88.72) but lower MaxEnt score (7.72 vs 5.4). Change of either c.-6-41g>c or c.-6-40t>c results in higher HSF matrics scores than the exon 2 SA. Change of c.-6-40t>c lowered the MaxEnt score to 4.7, however, the MaxEnt score for c.-6-41g>c and c.-6-41_40gt>cc increased and approaching the MaxEnt score of the *FOLR3* RefSeq exon 2 SA.

A. HSF matrics

Splice site	Motif	New splice site	Wild-type	Mutation	% variation
Exon 2 SA	Gctctctggcag GAA	–	87.52	–	–
g>c	ccccct**G**tctcaggcc	ccccct**C**tctcag GCC	88.72	91.34	2.95
t>c	cccctg**T**ctcaggcc	cccctg**C**ctcag GCC	88.72	89.38	0.74
gt>cc	ccccct**GT**ctcaggcc	ccccct**CC**ctcag GCC	88.72	**92.00**	3.69

B. MaxEnt

Type	Reference motif	Reference score	Mutation motif	Mutation score	% variation
Exon 2 SA	Tgactgtggctctctggcag GAA	7.72	–	–	–
g>c	ggctgttgcccct**G**tctcaggcc	5.4	ggctgttgcccct**C**tctcag GCC	6.85	26.85
t>c	ggctgttgcccctg**T**ctcaggcc	5.4	ggctgttgcccctg**C**ctcag GCC	4.71	−12.78
gt>cc	ggctgttgcccct**GT**ctcaggcc	5.4	ggctgttgcccct**CC**ctcag GCC	**7.11**	31.67

Table III

Very rare SNPs identified in myelomeningocele subjects.

SNPs	cDNA Change	Significance	A2 Frequency	Ref A2 Frequency	Method
SLC19A1					
rs527457211	c.-153-1832T>C	5′-promoter	0/0.003	0/0≠	WES
rs575838018	c.-153-1647T>G	5′-promoter	0/0.003	0/0≠	WES
rs577772783	c.-50+935C>T	intron	0/0.003	0/0≠	WES
rs529380110	c.-50+1375C>T	intron	0/0.003	0/0≠	WES
rs547611896	c.-50+1787G>T	intron	0/0.003	0/0≠	WES
rs564067188	c.-49-1417G>T	intron	0/0.003	0.007/0.008≠	WES
rs776875455	c.189+1974C>T	intron	0/0.003	ND	WES
rs576000521	c.189+2601C>T	intron	0/0.003	0/0≠	WES
rs1051269	c.246C>G	silence	0.01/0.0	0.01/0.006≠	Sanger
rs760930392	c.532G>A	p.G178S	0/0.003	0.0004†	WES
rs756426597	c.584T>A	p.F195Y	0/0.003	0.00015†	WES
rs9282853	c.784C>A	silence	0.01/0.0	0.007/0.001≠	Sanger
rs9282854	c.786G>A	silence	0.0/0.01	0.00016†	Sanger
rs779086468	c.924G>A	silence	0/0.003	0.00002†	WES
rs200236009	c.941C>T	p.T314M	0.01/0.0	0.001/0.0≠	Sanger
rs200647386	c.971C>T	p.A324V	0.01/0.04	0.0006†	Sanger
rs773969659	c.1137C>G	silence	0/0.003	0.00003†	WES
rs752889476	c.1151+39C>T	intron	0.01/0.0	0.00001†	Sanger
rs2039276	c.1152-97C>A	intron	0.03/0.01	0.017/0.007≠	Sanger
rs73228786	c.2281-143A>G, c.*+363A>G	intron/3′-UTR	0.02/0.0	0.030/0≠	Sanger
rs141599346	c.1428C>T, c.*640C>T	intron/3′-UTR	0.02/0.0	0.005/0≠	Sanger
FOLR1					
rs5792580	c.-74-158delC	intron	0.0238/0.0238	0/0≠	Sanger

SNPs	cDNA Change	Significance	A2 Frequency	Ref A2 Frequency	Method
FOLR2					
rs141864268	c.-21C>T	5′-UTR	0/0.0120	0.0060/0[≠]	Sanger
rs140123489		syn/p.D81D	0.0100/0	ND	Sanger
FOLR3					
rs193208888		syn/p.V199V	0.0110/0	0.0050/ND[≠]	Sanger
rs200896195		stop gain/p.R204X	0.01/0	0.0006/ND[≠]	Sanger

Note: A2, rare variant allele;

[*] represents stop codon of cDNA; syn, synonymous change, position of allele in human chromosome 11 with reference to human genome sequences hg19. Rare allele A2 frequency (Caucasian/Hispanic Mexican MM subjects) for SNP with reference to

[†] EVA-ExAc_aggregated from multiple studies listed http://exac.broadinstitute.org/faq [Total of 60,706 individuals: African/African American (5,203), Latino (5,789), East Asian (4,327), South Asian (8,256), Finnish (3,307), Non-Finnish European (33,370), Other (454)], or

[≠] 1KGenomes A2 frequency for EUR/MXL. ND – population data not available.

Table IV

Allele frequency of common genetic variants (SNP) identified among 96 MM subjects. Note: 1KGenomes A2%, A2 allele frequency of EUR/MXL populations; Cauc, Caucasian; Hisp, Hispanic; A1, reference common allele; A2, rare variant allele; MM, myelomeningocele; ND, no data for reference population; syn, synonymous change, position of allele in human chromosome 11 with reference to human genome sequences hg19. Fisher's exact test p value (2 tailed) and the p<0.05 is bolded.

			Caucasian A2% (A2/A1)			Mexican American A2% (A2/A1)		
SNP ID	**Chr Loc**	**Significance**	**MM**	**NHLBI EA/*CEU**	***p*-value**	**MM**	**1KGenomes MXL**	***p*-value**
SLC19A1								
rs150492570	46958003	intron	36.7% (22/38) (36.7	56.6% (112/86)	**0.0080**	34.9% (30/56)	64.8% (83/45)	**0.0001**
rs13052294	46957982	intron	64.8% (57/31)	57.1% (113/85)	0.2419	62.8% (49/29)	64.8% (83/45)	0.7672
rs1131596	46957916	5′-UTR	61.5% (59/37)	57.1% (113/85)	0.5286	62.5% (60/36)	64.8% (83/45)8	0.7791
rs1051266	46957794	p.H27R	62.5% (60/36)	57.1% (113/85)	0.4484	62.5% (60/36)	64.8% (83/45)	0.7791
rs12659	46951556	silence	64.6% (62/34)	58.1% (115/83)	0.3110	62.5% (60/36)	65.6% (84/44)	0.6735
rs35978794	46935342	intron/3′-UTR	37.2% (35/59)	40.4% (80/118)	0.7007	39.6% (38/58)	36.7% (47/81)	0.6785
rs11284347	46935078	3′-UTR	17.0% (16/78)	46.0% (91/107)	**0.0001**	20.2% (19/75)	42.2% (54/74)	**0.0008**
rs1051296	46934861	3′-UTR	37.5% (36/60)	40.4% (80/118)	0.7030	39.1% (36/56)	35.9% (46/82)	0.6726
rs1051298	46934826	3′-UTR	37.5% (36/60)	40.4% (80/118)	0.7030	39.1% (36/56)	35.0% (46/82)	0.6726
FOLR1								
rs2071010	71900964	5′-UTR	7.45% (7/87)	5.79% (11/187)	0.6040	7.45% (7/87)	5.79% (11/187)	0.6040
rs9282688	71901462	intron	1.25% (1/79)	3.53% (7/191)	0.4457	1.25% (1/79)	3.53% (7/191)	0.4457
FOLR2								
rs651646†	71929526	intron c.-24-79A>T	44.4% (40/50)	42.4% (72/98)	0.7929	33.3% (28/56)	47.7% (63/69)	0.479
rs2298444†	71932414	intron c.475+59T>C	28.1% (27/69)	18.8% (32/138)	0.0916	18.8% (18/78)	27.3% (36/96)	0.1568
FOLR3								
rs1802609	71847080	syn (p.R28R)	6.3% (6/90)	4.6% (395/8191)	0.8011	1.0% (1/95)	1.5% (2/130)	1.000
rs61734430	71550130	non-syn (p.R98C)	3.1% (3/93)	5.3% (456/8130)	0.4897	0.0% (0/96)	1.5% (2/130)	0.5103
rs139130389	71850156	stop-loss	11.5% (11/85)	7.3% (600/7654)	0.1155	0.0% (0/96)	4.5% (6/126)	**0.0409**
rs79284835	71850453	syn (p.E140E)	0.0% (0/96)	0.04% (3/8539)	1.0000	3.1% (3/93)	3.8% (5/127)	1.000
rs116309149	71850589	intron c.493+72G>A	3.1% (3/93)	2.6% (225/8355)	0.7414	0.0% (0/96)	2.3% (3/129)	0.2655

			Caucasian A2% (A2/A1)			Mexican American A2% (A2/A1)		
SNP ID	**Chr Loc**	**Significance**	**MM**	**NHLBI EA/*CEU**	***p*-value**	**MM**	**1KGenomes MXL**	***p*-value**
rs1802608†	71850722	syn (p.H195H)	12.5% (12/84)	6.3% (544/8040)	**0.0320**	2.1% (2/94)	2.3% (3/129)	1.000

*CEU are Utah residents with northern and western European ancestry used for the HapMap project and the 1000 Genomes Project (1KGP), NHLBI EA are European American from in the National Heart, Lung, and Blood Institute Exome Sequencing Project. MXL are Americans of Mexican ancestry living in Los Angeles in the 1000 Genomes Project (1KGP).

†NIEHS SNP Function Prediction FuncPred (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm) predicts the SNP is located within a transcription factor binding site or linked to another SNPs located within a transcription binding site or splicing enhancer/silencer.

Table V

NTD GG allele frequency of previously published studies by FA fortification policy in each country of origin at the time of enrollment.

Author (year)	Country of origin	FA fortification policy at time of enrollment	NTD GG allele frequency	Studies by FA fortification policy	*p-value*
De Marco et al. (2003)	Italy	Voluntary *	0.32 (46/144)		
Relton et al. (2004)	United Kingdom	Voluntary *	0.33 (67/206)	0.301 (185/614)	
O'Leary (2006)	Ireland	Voluntary *	0.27 (72/264)		**0.0468**
Shaw et al. (2002)	United States	No fortification	0.28 (37/132)		
Pei et al. (2014)	China	No fortification †	0.45 (44/98)	0.365 (119/326)	
Findley et al. (2017)	United States	No fortification	0.40 (38/96)		

*From Flynn A et al., 2009.

†From Ren A, 2015.