Characteristics of a Spina Bifida Population Including North American Caucasian and Hispanic Individuals

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BACKGROUND: Meningomyelocele (MM) is a common human birth defect. MM is a disorder of neural development caused by contributions from genes and environmental factors that result in the NTD and lead to a spectrum of physical and neurocognitive phenotypes. **METHODS:** A multidisciplinary approach has been taken to develop a comprehensive understanding of MM through collaborative efforts from investigators specializing in genetics, development, brain imaging, and neurocognitive outcome. Patients have been recruited from five different sites: Houston and the Texas-Mexico border area; Toronto, Canada; Los Angeles, California; and Lexington, Kentucky. Genetic risk factors for MM have been assessed by genotyping and association testing using the transmission disequilibrium test. **RESULTS:** A total of 509 affected child/parent trios and 309 affected child/parent duos have been enrolled to date for genetic association studies. Subsets of the patients have also been enrolled for studies assessing development, brain imaging, and neurocognitive outcomes. The study recruited two major ethnic groups, with 45.9% Hispanics of Mexican descent and 36.2% North American Caucasians of European descent. The remaining patients are African-American, South and Central American, Native American, and Asian. Studies of this group of patients have already discovered distinct corpus callosum morphology and neurocognitive deficits that associate with MM. We have identified maternal MTHFR 667T allele as a risk factor for MM. In addition, we also found that several genes for glucose transport and metabolism are potential risk factors for MM. **CONCLUSIONS:** The enrolled patient population provides a valuable resource for elucidating the disease characteristics and mechanisms for MM development. *Birth Defects Research (Part A) 82:692–700, 2008.* © 2008 Wiley-Liss, Inc.

Key words: meningomyelocele (MM); Caucasian; Hispanic; brain morphology; neurocognition

INTRODUCTION

One in 33 babies born in the United States has a major birth defect. NTDs are a group of common birth defects with a congenital malformation of the CNS affecting approximately 6 in 10,000 livebirths in the United States (Yen et al., 1992) in 1989, which decreased to 5.4 in 2003–2004 (Boulet et al., 2008). The estimated spina bifida (SB; a subset of NTDs involving abnormalities of spinal closure below the region of the head) prevalence was reported at approximately 3.39 per 10,000 livebirths in the US during 2003–2004, decreased from 4.89 per 10,000 livebirths in 1995–1996 before folic acid (FA) fortification of enriched flour products was mandated by USFDA (Canfield et al., 2005; Boulet et al., 2008).

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NTDs basically result from a failure of the neural tube to close properly during the first month of pregnancy, a period when the pregnancy most often has not yet been recognized by the mother. While NTDs broadly include lack of closure anywhere along the neural tube, the majority of cases can be categorized as either lack of closure in the region of the head (anencephaly) or lack of closure below the head (SB, also termed "split spine") with approximately equal frequencies observed at birth (Botto et al., 1999; Melvin et al., 2000). Individuals affected with anencephaly die soon after birth. With modern medical treatment, nearly all babies born with SB survive. The most common form of SB is meningomyelocele (MM; lack of closure along the spine affecting the meninges as well as the neural tissue). Surgical closure must occur within 48 h of birth to prevent infection and further damage (Walsh and Adzick, 2003). Many patients require ventriculo-peritoneal shunting because of the associated hydrocephalus. Depending on level of spinal defect, the child may never walk or achieve bowel or bladder control. Additionally, there are learning difficulties, only recently appreciated (Fletcher et al., 1992b, 2002, 2005).

The physical and intellectual handicaps associated with NTDs can range from mild (in a minority of cases) to severe (in many cases), resulting in morbidity and sometimes premature mortality for affected individuals as well as a significant societal burden (Iqbal, 2000). An estimated total annual economic cost of $489 million dollars per year (in 1992 dollars) was suggested by a year 2000 study (Iqbal, 2000) to provide physical and rehabilitation care for all SB patients in the US. As the most common severely disabling birth defect in North America, our knowledge of SB is very fragmented. Extensive research ranging from large epidemiological studies, creating rodent models, and gene association studies have been conducted trying to determine the molecular underpinnings leading to formation of NTDs. Others have studied the neurocognitive function deficits specifically observed in MM patients in order to understand the core deficits among these patients (Dennis et al., 1981, 1993; Brandt et al., 1994; Landry et al., 1995; Fletcher et al., 1992a,b, 1996). MM is a disorder of neural development with contributions by complex gene and environmental factors interacting to produce the NTD and leading to a spectrum of physical and neurocognitive phenotypes. Comprehensive understanding of MM requires a multidisciplinary approach.

History of Project

A collaborative effort from investigators specializing in genetics, development, brain imaging, and neurocognitive outcome was initiated at our institution (The University of Texas Medical School at Houston) in 1997 to set up a Program Project with Dr. Jack Fletcher as principal investigator. Our main goal was, and continues to be, integration of knowledge learned by different investigators who are studying the same cohort of MM-affected individuals to aid in prevention and treatment of this birth defect. We seek to elucidate physical and neural mechanisms underlying the variability in outcomes, and the genetic and environmental factors underlying this variability in the phenotype of MM. The Program Project includes projects with aims to: (a) evaluate genetic factors associated with the physical, neural, and behavioral/cognitive phenotypes; (b) characterize the physical, neural, and cognitive/behavioral phenotypes; (c) evaluate core process and functional deficits across life-span from infancy and middle adulthood; (d) provide specific evaluations of the role of the CNS anomalies of the spine and brain associated with MM to the phenotypes.

One focus of the Program Project is to determine genetic factors involved in MM formation. Many mouse models provide evidence to support the important role of genes in NTD development (Harris and Juriloff, 2007). There exists variation of disease prevalence along ethnic and racial lines (e.g., Irish); having a much higher *a priori* risk to have a child with NTD also infers genetic etiology of NTD (Melvin et al., 2000). While there are many clues from previous studies, the work to define the genetic components causing MM is hampered by the complex genetic nature of the problem. Closure of the neural tube is an extremely intricate process requiring the appropriate work of many different genes. We are dealing with no clear-cut mode of inheritance and few families with more than one affected person. Fortunately, many affected families are interested in participating in research to try and help unlock the mystery.

With many new genetic techniques and the completion of the Human Genome Project providing vast knowledge of new genetic markers, we are using these new tools to examine genetic markers for associations with risk for MM in our patients. Our approach utilizes simplex MM families (families with one member affected with an MM) in the genetic studies using the transmission disequilibrium test (TDT) statistical approach to identify risk associating genes in concert with effects of environmental influences. To aid in sorting out the environmental influences affecting the genetic factors, we are obtaining data from two detailed surveys assessing socio-demographic, epidemiologic, and environmental factors.

For the genetic studies, we have chosen to recruit from two major ethnic groups in North America: Hispanics of Mexican descent and Caucasians of European descent. In North America, individuals of Hispanic Mexican heritage have the highest risk of having NTD-affected offspring (Canfield et al., 1996a,b), prompting our project to select Hispanic Mexican-Americans as one of the two major populations to study other than the Caucasian-Americans of European descent. The Hispanic population is the largest and fastest growing minority in the US, making NTDs in Hispanics a major public health issue. Unfortunately, genetic studies of Hispanic individuals in North America are more difficult than study of individuals from other ethnic groups because of the complexity. Many studies have reported the admixed nature of Mexican-Americans in Texas and surrounding border states (Hanis et al., 1991; Bertoni et al., 2003; Price et al., 2007). Using ethnic specific single nucleotide polymorphism (SNP) markers, it was found that the major contributing ancestries for Hispanic- Americans in the Southern and Western US are European and Native American with a small percentage African ancestry. A higher contribution of African ancestry for Hispanic-Americans in the North and Northeastern US has been reported (Bertoni et al., 2003). Defining the ancestral contribution of admixed population assists in selection of appropriate controls and genetic markers for genetic association studies. Determining admixture in our Hispanic patients, parents, and controls is an important goal of the study with results reported below.

The following sections describe the properties of the MM patient population in our study along with a description of our approach to identify the genetic and environmental factors contributing to the risk of developing MM.

MATERIALS AND METHODS

Patient Recruitment

Patients and their parents were enrolled in the study after obtaining informed consent and their blood and/or saliva samples were sent to our laboratory for DNA extraction. The protocol of this study was approved by the Committees for the Protection of Human Subjects at The University of Texas Medical School at Houston and Baylor College of Medicine. Sites for enrollment of subjects born between 1955 and 2008 (ages 0.4–53 years) and their parents included the Shriners Hospital for Children at Houston (HOU), Texas; the Shriners Hospital for Children at Los Angeles (LA), California; the Shriners Hospital for Children at Lexington (LEX), Kentucky; Texas Children's Hospital (TCH), Houston, Texas; Hospital for Sick Children, Toronto (TOR), Canada; and individuals referred from Texas not treated at HOU and TCH (OTH). Patients were recruited by nurses at clinic visits or at support group meetings. Patients with a diagnosis of an isolated (nonsyndromic) MM at birth were eligible. The baseline information gathered on each study subject included ethnicity, level of defect, and family history.

The ethnicity of the affected individual was determined according to: (1) a statement by parent that the parent is a member of a specific ethnic group; (2) place of birth of parent; and/or (3) statement that the parent is a descendent of a specific ethnic group. The level of defect was determined from the medical record and in some cases by review of X-rays. Level of defect was classified according to the vertebral sites that failed to close resulting in formation of MM (i.e., at or above vertebrae L-1 and at or below vertebrae L-2). A three-generation family history was obtained along with information regarding any more distantly related individuals who had any features suggestive of/consistent with an NTD of any type.

Sample Preparation

Blood samples and/or saliva samples were obtained from the patients and both parents when possible. Genomic DNA from blood cells was extracted using the Puregene DNA extraction kit (Gentra Systems, Inc., Minneapolis, MN) and Oragene DNA collection kit (DNA Genotek, Inc., Ottawa, Ontario, Canada) from saliva. Anonymous control DNAs from 92 Hispanic individuals from the Houston area and 92 Caucasian individuals from the HD100CAU panel were used as negative controls. Genotyping quality control was accomplished using DNA samples from 30 CEPH families used in HapMap project. Patient family DNAs from Toronto, Canada were extracted before sending to our laboratory.

Survey/Questionaire

One goal of our Program Project is to analyze the joint effects of environmental factors and genetic variants of MM candidate genes in determining the phenotypes of our MM patients including lesion level, brain dysmorphologies, cognitive function, and behavior. We designed two surveys to collect information on socio-demographic, epidemiologic, and environmental factors including dietary status of our patient families. The environmental survey questions will be posted on the web link http://www.uh.edu/~sandi/. In addition, participants were asked to complete a highly detailed nutrition survey on dietary habits, designed with NutriQuest. The nutrition survey consists of questions ranging from general habits to specific questions about several foods within a variety of different categories (e.g., beverages, fruits, vegetables, grains, meat, and dairy). For each specific food, participants were asked both frequency and quantity consumed.

Genetic Marker Selection

A candidate gene approach was designed for our study with the focus on genes involved in the folate metabolism/catabolism pathways (Volcik et al., 2000), glucose homeostasis maintenance (Davidson et al., 2008), and genes known to cause NTDs in small animals when mutated (Harris and Juriloff, 2007; Volcik et al., 2002). Lists of genes and genomic regions known to associate with SB were selected through literature review. Microsatellite markers and SNPs within or near the genes of interest were obtained from public databases (http://www.ncbi.nlm.nih.gov/SNP/ and http://genome.cse.ucsc.edu/) with SNP heterozygosity present in at least 5% of the HapMap CEU (Centre d Etude du Polymorphisme Humain UTAH residents with ancestry from Northern and Western Europe) population. SNPs with potential functional implication were preferentially selected. The information on the heterozygosities of almost all selected SNPs for Mexican-Americans was not publicly available at the time of SNP selection and only limited numbers of SNPs are available at present. For follow-up evaluation, a SNP screening density of ~1 Kb per SNP was performed for genes associated with significant SNPs in an initial low density screen.

DNA Genotyping

SNP genotyping was carried out using the SNPlex Genotyping platform (ABI, Foster City, CA) based on an oligonucleotide ligation/PCR/probe hybridization assay that can interrogate selected 48 SNPs simultaneously in one reaction. Working DNA stocks of 200 ng were used for each SNPlex reaction. PCR amplification was performed in a 10 μL reaction volume. The reaction experiments were performed using the manufacturer's standard SNPlex protocol with the raw genotyping runs using the ABI 3730xl DNA analyzer. Data analyses were performed using the GeneMapper v4.0 software and the genotypes called by the software were examined by at least two investigators before exporting and compiling for statistical analyses.

Admixture Analyses

Genotypes generated on 23 SNPs in 12 genes located on nine different chromosomes were used to determine the proportion of Yoruban African (YRI) versus CEU ancestry in the Caucasian (n = 230) and Hispanic-Mexican (n = 336) MM patient populations using the program Structure (Pritchard et al., 2000; Falush et al., 2003, 2007).

Table 1
Ethnicity, Family Structure, and Gender Distribution of MM Patients Recruited from Different Sites

	Patient Recruiting Sites						
	HOU	TCH	TOR	LA	LEX	OTH	Total
Ethnicity							
Hispanic (Mex)	105 (12.1)	170 (19.7)	0 (0.0)	115 (13.3)	1 (0.1)	6 (0.7)	397 (45.9)
Caucasian	40 (4.6)	57 (6.6)	103 (11.9)	15 (1.7)	70 (8.1)	28 (3.2)	313 (36.2)
African-American	11 (1.3)	22 (2.5)	2 (0.2)	0 (0.0)	0 (0.0)	0 (0.0)	35 (4.0)
Asian/Pac	3 (0.3)	0 (0.0)	3 (0.3)	0 (0.0)	0 (0.0)	0 (0.0)	6 (0.7)
Hispanic (non-Mex)	1 (0.1)	10 (1.2)	0 (0.0)	11 (1.3)	0 (0.0)	0 (0.0)	22 (2.5)
Others	5 (0.6)	30 (3.5)	8 (0.9)	2 (0.2)	0 (0.0)	1 (0.1)	46 (5.3)
Unknown	10 (1.2)	14 (1.6)	13 (1.5)	2 (0.2)	0 (0.0)	7 (0.8)	46 (5.3)
Total	175 (20.2)	303 (35.0)	129 (14.9)	145 (16.8)	71 (8.2)	42 (4.9)	865 (100.0)
Family structure							
Trio	91 (10.5)	196 (22.7)	85 (9.8)	82 (9.5)	27 (3.1)	28 (3.2)	509 (58.8)
Mother/child	63 (7.3)	98 (11.3)	35 (4.0)	48 (5.5)	31 (3.6)	12 (1.4)	287 (33.2)
Father/child	3 (0.3)	5 (0.6)	2 (0.2)	6 (0.7)	6 (0.7)	0 (0.0)	22 (2.5)
Child	19 (2.2)	3 (0.3)	7 (0.8)	9 (1.0)	7 (0.8)	2 (0.2)	47 (5.4)
Total	176 (20.3)	302 (34.9)	129 (14.9)	145 (16.8)	71 (8.2)	42 (4.9)	865 (100.0)
Gender							
Female	89 (10.3)	155 (17.9)	61 (7.1)	75 (8.7)	36 (4.2)	20 (2.3)	436 (50.4)
Male	79 (9.1)	147 (17.0)	59 (6.8)	60 (6.9)	29 (3.4)	19 (2.2)	393 (45.4)
Unknown	7 (0.8)	1 (0.1)	9 (1.0)	10 (1.2)	6 (0.7)	3 (0.3)	36 (4.2)
Total	175 (20.2)	303 (35.0)	129 (14.9)	145 (16.8)	71 (8.2)	42 (4.9)	865 (100.0)

HOU, Shriners Hospital for Children at Houston, Texas; TCH, Texas Children's Hospital, Houston, Texas; TOR, The Hospital for Sick Children Toronto, Canada; LA, Shriners Hospital for Children at Los Angeles, California; LEX, Shriners Hospital for Children at Lexington, Kentucky; OTH, referrals from Houston and Texas border areas. Hispanic (Mex), Hispanics of Mexican-American descent; Hispanic (non-Mex), non-Mexican Hispanics; Asian/Pac, Asian and Pacific Islander; "Others" includes Native American and other country of origin. Unknown ethnicity, ethnicity information not available. Unknown gender, gender information not available. Data present in number of families followed by percentages in parentheses with reference to the total number of families in the study.

The 23 SNPs included: rs11761556, rs11763517, rs1188977, rs12406072, rs1286648, rs1286763, rs1286765, rs1385068, rs1435706, rs1465057, rs17016566, rs2051423, rs2715553, rs2850760, rs2850763, rs3754219, rs3818569, rs3828942, rs4998557, rs697763, rs743682, rs7487904, and rs799917. These loci were selected based on an average difference in minor allele frequencies of 0.3 or higher between the YRI and the CEU populations of the HapMap. A proportion of YRI and CEU ancestry of each individual sample was computed with 5,000 burn-in periods for 10,000 repetitions and data were plotted in relationship to the YRI and CEU reference clusters.

Statistical Analysis

We chose to use the TDT in GeneHunter2 (Kruglyak et al., 1996) to test association of genetic markers with risk of MM because: (1) almost all of the MM patient families were small trios, and (2) more then half of our patients were Hispanics of Mexican descent. An important feature of the TDT is its robustness in studying populations with substructures like the MM patient population we enrolled (Ewens and Spielman, 1995). To fully utilize genotype data from our sample set with a significant number of duos, we also used the reconstruction combined-TDT (Knapp, 1999a,b). To analyze quantitative phenotypes, we used quantitative-TDT (Abecasis et al., 2000). In addition to TDT, we also used the genomic control method (Devlin and Roeder, 1999) as a secondary method to analyze our data. The genomic control method uses data sets with no information on genealogy of the population and corrects for population heterogeneity, poor choice of controls, and cryptic relatedness of cases. A positive finding is concluded when the allele-wise and genotype-wise testing yields a p value less than .05 and the goodness-of-fit testing yields a p value greater than .05. Both Bonferroni correction and Monte Carlo approach were used to evaluate spurious significance of association due to multiple statistical tests. Interactions of gene-gene and gene-environment were examined using a nonparametric, model-free multifactor dimensionality reduction method (Ritchie et al., 2003). Analyses on the proportion of admixture in our patient subpopulations were performed using the "Structure" program (Pritchard et al., 2000; Falush et al., 2003, 2007).

RESULTS

Characteristics of Patients in the Study

Geographic locations. The patients in our study were enrolled from five major sites (Table 1). Approximately 60% of the patients were recruited in the Houston and Texas border areas and the remaining 40% were recruited from the LA area (16.8%), the Toronto area (14.9%), and Lexington area (8.2%). Approximately 69% of our Hispanic patients of Mexican descent were recruited in the Houston and Texas border areas and 29% from the LA area. Forty percent (125/313) of our Caucasian patients were recruited in the Houston and Texas border area (HOU, TCH, and OTH), 33% (103/313) from Toronto, 22% (70/313) from LEX, and 5% (15/313) from LA.

Family structure. To date we have recruited 509 trios, 287 mother/child duos, 22 father/child duos, and 47 affected individuals in our study to search for genetic factors contributing to MM (Table 1). The target for the study is to recruit 300 Hispanic trios of Mexican descent and 250 North American Caucasian trios of European

Table 2
Gender and Family Structure of MM Patients in Different Ethnic Groups

	Ethnicity of patient families							
	Hispanic (Mex)	Caucasian	African-American	Asian/Pac	Hispanic (non-Mex)	Others	Unknown	Total
Gender								
Female	204 (23.6)	162 (18.7)	19 (2.2)	3 (0.3)	11 (1.3)	22 (2.5)	15 (1.7)	436 (50.4)
Male	182 (21.0)	136 (15.7)	14 (1.6)	3 (0.3)	10 (1.2)	23 (2.7)	25 (2.9)	393 (45.4)
Unknown	11 (1.3)	15 (1.7)	2 (0.2)	0 (0.0)	1 (0.1)	1 (0.1)	6 (0.7)	36 (4.2)
Total	397 (45.9)	313 (36.2)	35 (4.0)	6 (0.7)	22 (2.5)	46 (5.3)	46 (5.3)	865 (100.0)
Family structure								
Trio	246 (28.4)	190 (22.0)	13 (1.5)	3 (0.3)	10 (1.2)	25 (2.9)	22 (2.5)	509 (58.8)
Mom/child	129 (14.9)	90 (10.4)	19 (2.2)	2 (0.2)	11 (1.3)	17 (2.0)	19 (2.2)	287 (33.2)
Father/child	4 (0.5)	11 (1.3)	2 (0.2)	1 (0.1)	1 (0.1)	2 (0.2)	1 (0.1)	22 (2.5)
Child	18 (2.1)	22 (2.5)	1 (0.1)	0 (0.0)	0 (0.0)	2 (0.2)	4 (0.4)	47 (5.4)
Total	397 (45.9)	313 (36.2)	35 (4.0)	6 (0.7)	22 (2.5)	46 (5.3)	46 (5.3)	865 (100.0)

Hispanic (Mex), Hispanics of Mexican-American descent; Hispanic (non-Mex), non-Mexican Hispanics; Asian/Pac, Asian and Pacific Islander; "Others" includes Native American and other country of origin. Unknown gender, gender information not available. Data represent number of patient families followed by percentages in parentheses with reference to the total number of families in the study.

descent. Currently we have recruited 246 Hispanic- Mexican trios and 190 North American Caucasian trios of European descent (Table 2). In 90% of the families recruited for the study, the affected individual is the only affected person; thus, in the vast majority of our recruited families there is a negative family history for NTDs. The remaining 10% of our families have reported to have either a close or distant relative with some form of NTDs and/or NTD-related complication (hydrocephalus, SB [either MM, meningocele, or SB occulta], or anencephaly). A similar proportion of families having a history of NTDs was present at each of the five recruiting sites (data not shown). One family had identical twins both affected with MM and two other families had fraternal twins, one set with both children affected and the other set discordant for MM.

Gender. Overall, our patient group had a slightly higher (50.4%) number of female affected patients than male patients (45.4%) and this trend was observed throughout patients of all ethnic groups and across all recruiting centers in our study (Table 1). The observed differences between numbers of female and male MM patients in the project and between sites was not statistically significantly different (Table 2) ($p = 1.0$; $\chi^2 = 1.0$;, degree of freedom = 6). A trend of more female patients among groups of different ethnicities was observed but the differences, again, were not significant (Table 2) ($p = .6$; $\chi^2 = 4.6$; degree of freedom = 6).

Ethnicity. This cohort of 865 MM patients consisted of two major ethnicities: Hispanics of Mexican descent (45.9%) and North American Caucasians of European descent (non-Hispanic whites, 36.2%) as seen in Table 2. Patients of other ethnicities included: 4.0% African-American, 2.5% Hispanic non-Mexican, 0.7% Asian/Pacific Islander, 5.3% others (Native American and others), and 5.3% of unknown ethnicity.

Admixture. We sought to determine how the proportion of admixture differed among the Caucasian MM patients, the Hispanic-Mexican MM patients, and the locally recruited Hispanic controls in our study. Analyses of the genotypes generated on 23 SNPs in 12 genes located on nine different chromosomes using the program Structure showed the YRI and CEU populations clustered nicely into either the first inferred cluster or the second inferred cluster, respectively, while the MM patients were located near the CEU cluster (data not shown). The proportion of African ancestry for the CEU and the MM Caucasian patients was very similar (9.2% for CEU and 10.6% for MM Caucasian patients) and slightly higher among MM Hispanic-Mexican patients (13.8%). Similar results were observed when comparing the Hispanic patients from LA (10.8% YRI ancestry) and the Hispanic patients from the Houston-Texas area (12.1% YRI ancestry). These proportions were not appreciably different. When similar analyses were performed for MM Hispanic-Mexican patients and Hispanic controls the proportions of YRI ancestry are also very similar (12.1 and 14.2%, respectively).

Age. In general, our patients were born between 1955 and 2008. Based on the available dates of birth data we collected, our patients recruited at different sites had median ages between 12.9 to 22.2 years with the range between 0.4–53.0 years. Patients recruited from the TCH site were younger (median age 12.9 years) and over half of these patients were conceived after 1992. The median ages for patients recruited are shown in Table 3. The mean ages for patients recruited through different sites were as follows: HOU (14.9 years), TCH (12.3 years), TOR (21.4 years), LA (19.93), LEX (21.6 years), and OTH (17.3 years). All patients recruited in the Houston-Texas area were younger than 26 years old. The youngest patient recruited was 0.4 years at TCH while the oldest patient recruited was 53 years old from the Toronto site.

Lesion level. Our MM population was divided by lesion level for purposes of some analyses. These categories were: (1) failure of closure at L1 and above and (2) failure of closure at or below L2. Some of our patients were reported to have the lesion in the sacral region. For these patients, more refined classification was not available to determine exact involvement from the lower lumbar to the S2 regions. In our study, male patients were less frequent (9.2%) among the MM patients with higher lesions (located on or above vertebrae L1) than female patients (13.1%) (Table 4), while they were almost evenly distributed (female 30.2%, male 31.2%) in the group having lesions below vertebrae L1. Because our study

Table 3
Median Age (Range) Distributions of MM Patients

	All	Caucasian	Hispanic-Mexican	African-American	Others
HOU	16.2 (2.8–24.4)	16.4 (13.7–24.4)	16.6 (3.0–23.4)	18.9 (10.5–22.4)	12.1 (2.8–19.2)
TCH	12.9 (0.4–25.5)	17.3 (0.7–25.5)	14.7 (0.9–24.7)	15.9 (1.5–23.3)	6.5 (0.4–22.5)
TOR	20.0 (10.9–53.0)	20.5 (12.0–53.0)	NA	14.0 (14.0–14.0)	15.5 (10.9–42.6)
LA	20.3 (10.5–29.1)	18.1 (10.5–24.7)	20.3 (11.3–29.1)	NA	20.5 (12.4–31.1)
LEX	22.2 (10.8–30.3)	22.2 (10.8–30.3)	NA	NA	NA
OTH	18.5 (0.8–29.7)	20.5 (8.3–29.7)	16.1 (10.0–18.5)	NA	12.8 (0.8–19.1)

Not all patient information included dates of birth. Patient recruitment sites include: HOU, Shriners Hospital for Children at Houston, Texas; TCH, Texas Children's Hospital, Houston, Texas; TOR, The Hospital for Sick Children Toronto, Canada; LA, Shriners Hospital for Children at Los Angeles, California; LEX, Shriners Hospital for Children at Lexington, Kentucky; OTH, referrals from Houston and Texas border areas. Hispanic (Mex), Hispanics of Mexican-American descent; "Others" includes non-Mexican Hispanics, Asian and Pacific Islander, Native American, and American of other country of origin. Data represent median ages for each group followed by age ranges in parentheses.

includes patients born after FA fortification, this may be a factor causing the difference we have observed between our study and others. For our subject population, the distribution of defect levels was relatively similar among patients of all the ethnic backgrounds (Table 4). Approximately 25% (100/397) Hispanic- Mexican and African-American and 22% (69/313) Caucasian-American patients had lesions at or above vertebrae L1. Lesions on or below vertebrae L2 were determined to have occurred in ~68% (270/397) Hispanic-Mexican and African-American patients and 59.7% (187/313) Caucasian-American patients.

We found a slightly higher number of female patients (25.9%) with lesions at or above vertebrae L1 than male patients (20.3%), although this difference was not significantly different ($p = .071$). Lesions at or below vertebrae L2 were observed in 59.9% female patients and 68.7% male patients (Table 4).

Between 17–27% of MM patients recruited through our five major sites had lesions located on or above vertebrae L1 (Table 4). The frequency of lesion location did not significantly differ across four sites (~21–26%), with the exception being the Toronto site at 17.1%. However, lesion information was not available for approximately 42% of the Toronto patients, indicating that the Toronto data may have been biased. Missing lesion information accounted for less than 10% of patients recruited through the other four sites.

DISCUSSION

To date, we have recruited an MM population including 509 trios and 309 duos. The majority of our patients were from three areas (Texas, Toronto, and LA) where the general NTD epidemiology and FA supplementation knowledge and usage status for the women of child bearing age have been reported (Hendricks et al., 1999; Canfield et al., 2005, 2006; de Jong-van den Berg et al., 2005; Goldberg et al., 2006). We had several reasons for focusing on recruitment of Hispanics of Mexican descent along the Northern Mexican border with Texas and California:

Table 4
Characteristics of MM Lesion Levels

	L1 and above	L2 and below	Lesion levels Sacral	Unknown	Total
Gender					
Female	113 (13.1)	261 (30.2)	3 (0.3)	59 (6.8)	436 (50.4)
Male	80 (9.2)	270 (31.2)	2 (0.2)	41 (4.7)	393 (45.4)
Ethnicity					
Hispanic (Mex)	100 (11.6)	270 (31.2)	1 (0.1)	26 (3.0)	397 (45.9)
Caucasian	69 (8.0)	187 (21.6)	2 (0.2)	55 (6.4)	313 (36.2)
African-American	9 (1.0)	24 (2.8)	0 (0.0)	2 (0.2)	35 (4.0)
Others	22 (2.5)	69 (8.0)	2 (0.2)	27 (3.1)	120 (13.9)
Recruitment sites					
HOU	45 (5.2)	119 (13.8)	1 (0.1)	10 (1.2)	175 (20.2)
TCH	80 (9.2)	193 (22.3)	4 (0.5)	26 (3.0)	303 (35.0)
TOR	22 (2.5)	53 (6.1)	0 (0.0)	54 (6.2)	129 (14.9)
LA	30 (3.5)	108 (12.5)	0 (0.0)	7 (0.8)	145 (16.8)
LEX	19 (2.2)	51 (5.9)	0 (0.0)	1 (0.1)	71 (8.2)
OTH	4 (0.5)	26 (3.0)	0 (0.0)	12 (1.4)	42 (4.9)
Total	200 (23.1)	550 (63.6)	5 (0.6)	110 (12.7)	865 (100)

Lesion levels: L1 and above, lesion located on or above vertebrae L1; L2 and below, lesion located on or below vertebrae L2. Hispanic (Mex), Hispanics of Mexican-American descent; Hispanics (non-Mex), non-Mexican Hispanics; Asian/Pac, Asian and Pacific Islander; "Others" includes Native American and other country of origin. Data show the number of patients and the percentage of patients in the whole cohort. Patient recruitment sites include: HOU, Shriners Hospital for Children at Houston, Texas; TCH, Texas Children's Hospital, Houston, Texas; TOR, The Hospital for Sick Children Toronto, Canada; LA, Shriners Hospital for Children at Los Angeles, California; LEX, Shriners Hospital for Children at Lexington, Kentucky; OTH, referrals from Houston and Texas border areas. Data represent number of patient families followed by percentages in parentheses with reference to the total number of families in the study.

proximity to our location; this population is more likely to be affected with an NTD (Hendricks et al., 1999); and because there was some evidence to support more genetic homogeneity among this group of Hispanic individuals than other Hispanic individuals throughout the US (Hanis et al., 1991). We chose North American Caucasians of European descent because they have the second highest incidence of NTDs in the US and represent the majority of the population.

For purposes of our genetics studies, we estimated the power of using TDT to detect risk allele ($\gamma 1 = 2$) with a power of nearly $_1$ for gene frequency ≥ 0.1 using a trio population of 550 when the risk allele acts in either a multiplicative, additive, or dominant manner ($\alpha = 0.01$). A slight decrease in power to 0.9–0.97 was estimated when the number of trios was reduced to 300. The current MM population we recruited should provide sufficient power for detecting MM risk alleles with gene frequency higher than 0.1. In a recessive model, we estimated 550 families provide a power of 0.98 for a gene frequency of 0.5 ($\alpha = 0.01$), but the power deteriorated quickly to 0.1 with a gene frequency of 0.1. A much larger number of families will be needed to achieve statistical power over 0.8 if MM inherited in a recessive manner. In addition, a larger number of families will be needed to examine genetic factors with relative risk less than 2. The current number of trios and duos already provided us sufficient statistical power for TDT analyses and led us to further investigate other SNPs within the breast cancer gene 1 (*BRCA1*) (King et al., 2007), leptin receptor (*LEPR*), solute carriers type 2 A gene 1 (*SLC2A1* alias *GLUT1*), and hexokinase 1 gene (*HK1*) that showed association with MM risk in our patient group (Davidson et al., 2008). The MM population in our study has also been reported to show associations with methylene tetrahydrofolate reductase variant (*MTHFR* 677T) and microsatellite markers near the pairbox (*PAX1*, *7*, and *8*) genes (Volcik et al., 2000, 2002). Recruitment is continuing with the goal to obtain at least 300 Hispanic trios of Mexican descent and 250 North American Caucasian trios of European descent to improve the power of analyzing risk factors for individual ethnic groups and for subphenotype (i.e., brain dysmorphologies, neurocognitive deficits) analysis.

In 2006, the daily consumption of a supplement with FA among Texas women of child bearing age was determined to be low (40% non-Hispanic White, 24% Hispanic) (Canfield et al., 2006), especially for residents along the Mexican border (20.8%). The finding for usage of supplements containing FA among women in the Toronto area between 1998 and 2002 was similar (47.2% White, 17.9% Hispanic, respectively) and FA usage in the relevant period between 1988 and 1994 was 15–20% (de Jong-van den Berg et al., 2005). We do not anticipate the proportion of folate-resistant MM cases in our study patients to represent an overwhelming majority considering the low FA usage among Hispanics, especially along the Texas-Mexico border (Hendricks et al., 1999). The US Public Health Service recommended women of childbearing age to consume 400 μg FA daily starting in 1992 and the recommendation was quickly endorsed by the Institute of Medicine, American Academy of Pediatrics, and American College of Preventive Medicine. A mandate for food fortification with FA was made starting in 1998 by the USFDA. These recommendations and mandate need to be taken into account when analyzing data generated on our patient population. Patients in our study were born between 1955 and 2008. Over half of the patients recruited from the TCH site were conceived after 1992. Half or more of the patients recruited through different sites (HOU, TOR, LA, LEX, and OTH) were born before 1992. Therefore, we anticipate half or more of the patients in our study should not be affected by the US Public Health Service recommendation on FA. The impact of FA supplementation among patients 16 years and older should be even lower and, thus, among these patients we anticipate having a larger proportion who potentially have MM due to lack of FA. We are collecting information in two surveys to help us to delineate the effect of the mandated FA fortification by USFDA on our study.

We have shown by admixture analysis that our two patient groups, Hispanics of Mexican descent and North American Caucasians of European descent, are appropriately matched genetically with the controls groups we have recruited or obtained commercially. We were especially cognizant of the problems faced regarding genetic studies of individuals whose ethnicity is labeled as "Hispanic". Through our admixture studies we have shown that our Hispanic-Mexican sample including patients recruited in the areas of Houston, Texas, the Texas-Mexico border, and Los Angeles, California, is genetically the same with respect to their CEU and YRI ancestries. Genotypes for the SNPs we tested are not publicly available for Native American as for the CEU and YRI. We can, therefore, compare genotyping results from our patient sample with our recruited control sample with confidence that the comparison is valid. The similarity in contributions of European and African ancestry to Hispanic-Mexicans in Texas and LA has been reported previously (Price et al., 2007).

Anencephaly has been documented in multiple studies to be two to three times more common among females than males, but other NTDs including SB showed equal prevalence between both genders in studies published between 1989 and1991 (Canfield et al., 1996a,b). A study of SB patients in California between 1983 and1987 reported more male patients among a group having isolated high open defects and fewer male patients among the group including all closed defects (Shaw et al., 1994). In our study, female patients were more frequent among the MM patients with lesions located on or above vertebrae L1, while almost evenly distributed in the group having lesions below vertebrae L1. Our study includes patients born after the USFDA mandated FA fortification. Whether FA plays a role in the gender differences we have observed between our study and the previous study is not known. A difference in lesion definition between our study and the previous study could also be the reason for observed differences.

We are now gathering data regarding environmental factors that may be important in susceptibility to the formation of MM as well as variation in the MM phenotype. We are surveying our population for socio-demographic, epidemiologic, and environmental factors, following up on previously identified factors that have been implicated as important. We are gathering data on socioeconomic status, maternal and paternal ages and occupations, parental birthplaces, and maternal reproductive history (gravidity, parity, spontaneous abortions, birth order) including place of conception. Additionally, we are gathering environmental data including maternal diet and

vitamin use (folate and other micronutrients), maternal glucose status (diabetes/epilepsy), maternal exposure to hyperthermia (illness/hot tubs), maternal medication use (epilepsy, other illnesses), and maternal use of recreational substances (alcohol, tobacco and illicit drugs). As noted above, we are making efforts to account for folate status of the mothers dependent on year of the patient's birth regarding the recommendations for folate usage followed by USFDA-mandated FA fortification of enriched flour. By performing gene-environment analyses utilizing the data gathered from our surveys, we should be able to make more progress on both susceptibility and phenotypic variability in MM.

In addition, multiple neurocognitive functioning and brain imaging studies have been performed in some of the study population (Barnes et al., 2006; Burmeister et al., 2005; Dennis et al., 2001, 2002, 2004, 2005a,b,c, 2008; Edelstein et al., 2004; Fletcher et al., 2002, 2005; Hetherington et al., 2006; Huber-Okrainec et al., 2002, 2005; Lomax-Bream et al., 2007), making it possible to study the effect of genetics and the environment on neuroembryogenesis resulting in CNS dysmorphologies and subsequent neurocognitive deficits. From the study population, it was identified that over 50% of children with MM have part of both ends of the corpus callosum missing, a finding consistent with learning and behavioral deficits (Dennis et al., 2004; Fletcher et al., 2005). We plan to utilize the detailed phenotyping obtained in other projects of the Program Project to make correlations with the genetic variants.

In conclusion, the MM patient population in our study has reached a sufficient size to evaluate significance of risk alleles to MM development using standard and reconstruction-combined TDT. Over 50% of the enrolled patient trios were Hispanics of Mexican descent, a group known to have a higher incidence of MM. In addition to MM lesion level, other members of the Program Project are actively pursuing MM disease phenotypes, including brain dysmorphology and neurocognitive deficits, to expand our understanding of the diseases. Correlation of genetic and environmental factors to the newly delineated disease phenotypes will help us elucidate the physical and neural mechanisms for MM outcomes.

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